Molecular and biological investigation of *Pseudomonas aureofaciens* PA147-2 as a potential biocontrol agent of phytopathogenic fungi

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Af$^-$	antifungal minus
Af$^+$	antifungal plus
APase	alkaline phosphatase
Blast	basic local alignment search tool
bp	base pair
cfu	colony forming unit
CIAP	calf intestinal alkaline phosphatase
°C	degrees Celcius
dH_2O, sdH_2O	double distilled water, sterile double distilled water
ECL	enhanced chemiluminescence
FIGE	Field inversion gel electrophoresis
hr	hour
kb	kilobase
kDa	kilo dalton
LB	Luria Bertani medium
LTTR	LysR-type transcriptional regulator
M	molar
mA	milliamps
min	minute
OD_{xxx}	Optical density at a wavelength of xxx nm
ORF	open reading frame
PAGE	polyacrylamide gel electrophoresis
PGPR	Plant growth-promoting rhizo-bacteria
Phl	2, 4-diacetylphloroglucinol
Phz	Phenazine
Pi	inorganic phosphate
PMM	Pseudomonas minimal medium
rpm	revolutions per minute
SDS	sodium dodecyl sulphate
sec	second
SSC	sodium chloride sodium citrate
TEMED	N,N,N',N'-tetra-methylethylenediamine
TPDA	Tris buffered potato dextrose agar
w/v	weight per volume

Abstract

Pseudomonas aureofaciens PA147-2 can inhibit the *in vitro* growth of phytopathogenic fungi, and suppress *Phytophthora* rot of asparagus in the glasshouse and in the field.

Three antifungal defective mutants of PA147-2 were examined in detail. Two mutants, PA109 and PA138, were shown to have mutations in global regulator genes. The mutated gene in PA109 (designated *finT*) has similarity to members of the two-component regulator family, while PA138 has an insertion in a gene encoding a putative LysR-type transcriptional regulator (designated *finR*). FinR was found to be required *in cis* for the regulation of an adjacent gene (*finA*), and a mutation in *finA* resulted in a reduction of antifungal activity. Protein analysis indicated that FinR and FinT have both positive and negative regulatory effects, and the two regulators have common targets for regulation. The N-terminal sequence of a protein that is not expressed by *finR* and *finT* mutants was not similar to any known protein.

P. aureofaciens mutant PAH26 was shown to have an insertion in *pstA*. Consistent with findings in other organisms, the insertion in *pstA* conferred defects in high affinity phosphate uptake and interfered with regulation of the phosphate regulon. Results suggest that the inability of PAH26 to inhibit fungal growth is not directly related to known functions of PstA. Both PAH26 and PA109 are capable of fungal inhibition in phosphate excess conditions, implicating FinT in the phosphate-mediated regulation of antifungal activity.

The *recA* gene of PA147-2 was cloned, and a simple system to construct *recA* deletions was developed. Creation of *recA* mutants using this system will facilitate *trans* complementation experiments.

A preliminary field trial demonstrated that *P. aureofaciens* was able to protect asparagus from *Phytophthora* rot, and revealed that a high population of PA147-2 was not maintained in the field. *In vitro* experiments indicated that some antifungal-defective mutants have increased fitness relative to PA147-2, and such mutants in laboratory culture conditions can outcompete the parental strain.

Chapter 1

Biological control: Nature versus nature

1.1 Introduction.

Biology is about balances. With few conceivable exceptions, organisms evolve alongside those with whom they share an environment. Ecosystems are dynamic. The numerous species present may interact in a variety of fashions, from parasitism, antagonism, symbiosis to apparent altruism. The balance that is struck by these interactions contributes to the overall stability of the system, and the underlying state of flux. Disruption to ecosystems would be predicted to have serious consequences; one example of this is the removal of plant and animal species to make way for agriculture. Alteration of the natural equilibrium has left agricultural plants susceptible to a variety of maladies, which require control by further human intervention.

Biocontrol has been defined as the reduction of disease through the agency of one or more living organisms other than the host or man (Baker, 1968). In a manner of speaking, biological control is an attempt to alter the balance in an artificially created ecosystem (agriculture). Because agricultural practices tend to involve mono-culture of crop plants, the multiplication of a single pathogen could have dire consequences on the productivity of the plants. In a worst case scenario, a single pathogen could reduce an entire crop to nothing. The goal of biocontrol is to introduce a natural enemy of the pathogen in order to reduce its population, thus creating a system with desirable traits. In essence, biocontrol is the application of an organism to a system in an attempt to reduce the population of an undesirable organism, and ultimately favour the growth of a desirable organism (usually a crop plant). Thus, the phrase "nature versus nature" is an apt summation of the concept of biological control.

In this opening chapter it is my intention to review relevant literature pertaining to the role of bacteria in biological control, and to examine some examples of successful application. Clearly this is a large and active field of research, so an exhaustive review is not undertaken. Rather, examples are provided to highlight the progress made in biocontrol research in recent years. Given the subject of this thesis, the use of a bacterium to control

fungal disease, it seems reasonable to limit consideration to the control of soil-borne fungal pathogens. Since much current research is devoted to the mechanism of antibiotic production by biocontrol organisms, a major feature of this review will be the examination of the genetic basis for antibiotic production and regulation, focusing on five well-known examples. Environmental factors influencing biocontrol will be discussed. The introduction to the current study will survey the existing knowledge on *Pseudomonas aureofaciens* PA147-2, and outline the objectives of this work.

1.2 Why biological control?

Biological control is not a new concept. Successful experiments on the use of biocontrol to reduce crop diseases have been carried out since the early part of last century (cited in Baker, 1987), but its acceptance in agriculture has been slow. This may be due in part to the development of disease-resistant crops, but is probably largely the result of the tremendous success of chemical control strategies. However, more recent biocontrol research has utilised an integrative approach combining the power of molecular biology with biochemistry and plant pathology. This has coincided with an increasing realisation of the hazards associated with chemical pesticides, and a growing repugnance toward their use. Thus, the pursuit of biocontrol has been reinvigorated as people have come to recognise the potential benefits.

1.3 Disease suppressive soils.

Certain soils have a natural ability to prevent the development of disease. These soils are considered to be "suppressive", as opposed to "conducive" soils that do not prevent disease. While some soils are naturally suppressive, others can develop suppressive characteristics over time, particularly when they are consistently used in monoculture (Schroth & Hancock, 1982). There are biological and physical factors involved with disease suppression by soil, and these are considered below.

1.3.1 Soil composition and characteristics.

Numerous properties contribute to the description of a "soil", making soils difficult to characterise. They are produced by the weathering of rocks and minerals, and the nature of

these rocks and minerals contributes greatly to soil structure and composition. The formation of new minerals (clays) also has an influence on the structure of soil, and this in turn influences the composition of the biota in the soil. Biological activities of microbes and plants give soil further characteristic properties, and influence the organic content of soil. So, in addition to the minerals that originally contributed to the formation of soil, the interaction of physical and biological processes has a major impact on the nature of any given soil (Berger, 1965; Buckman & Brady, 1969). The complexity and range of soils make a proper treatment of the subject impractical here, so structural and physical aspects of soil as they relate to biocontrol will receive minor attention.

An example of the influence of soil structure.

Stutz et al (1989) investigated the composition of suppressive (MS1) and conducive (MC1) soils from the Morens region in Switzerland. As discussed below, disease suppression can sometimes be transferred from a suppressive to a conducive soil, by mixing of the suppressive and conducive soils. However, this was not observed with the conducive Morens soils. In their examination of this phenomenon, Stutz et al (1989) found that MS1 was composed mainly of vermiculitic clay, while MC1 was mainly illitic and smectitic. By making artificial soils from vermiculite and illite, it was shown that *P. fluorescens* CHA0 could contribute to suppression of black root rot of tobacco in the vermiculite but not illite clays. Furthermore, the vermiculite clay allowed the bacterium to colonise the tobacco roots while colonisation in illite was poor. Thus, the suppressivity of soil could be altered by changing minerals, demonstrating that the mineral composition of soils is an important consideration in biocontrol (Stutz et al., 1989).

Chemical nature of soil.

The pH of soil has been associated with its ability to suppress disease. However, there is no pH optimum at which all diseases are suppressed. Rather, the suppressive pH is likely to be related to other factors such as the growth parameters of the pathogen. For example, acidifying soil that normally suppressed carnation wilting caused by *Fusarium* correlated with increased carnation wilting, while in another experiment, increasing the pH led to increased radish infection in a *Rhizoctonia* suppressive soil (Liu & Baker, 1980; Scher & Baker, 1980). It is difficult to know whether pH is important because of a direct

relationship with the pathogen, or whether pH changes alter other soil factors such as the range of indigenous organisms, which might outcompete and suppress the pathogen. Regardless, pH is clearly important in disease suppression. Another chemical factor that may be of importance is salinity (Schroth & Hancock, 1982), but as with pH, the exact nature of its influence is difficult to discern.

1.3.2 Biological factors.

A number of observations regarding the characteristics of suppressive soils suggested that biological factors such as indigenous microbes are at least partly responsible for disease-controlling ability. For example, a conducive soil can be made suppressive by mixing in a small amount of suppressive soil, an observation that seems incompatible with a purely physical phenomenon. In addition, suppressive characteristics can be inactivated by heat treatment and fumigation, which suggests microbes could be involved in suppression, but are killed by the treatments. However, while compelling, the circumstantial nature of these observations is insufficient to draw strong conclusions. For this reason, the nature of suppression was investigated in a number of soil types. In a study on suppression of *Gaeumannomyces graminis*, eight fluorescent pseudomonads were isolated from suppressive soils that, when added to conducive soils, could suppress *G. graminis* on wheat as well as the original suppressive soil (Cook & Rovira, 1976). Two further illustrative examples are the studies on *Fusarium* and *Rhizoctonia* suppressive soils (Liu & Baker, 1980; Scher & Baker, 1980). In both of these studies, suppressive soils were shown to have populations of organisms that, when transferred to conducive soil, were able to induce disease suppression. In the case of *Fusarium* suppression the organisms in question were *Pseudomonas spp.* When these bacteria were introduced in sterile conducive soil (Fort Collins clay loam) and subjected to heat treatment, the population was shown to decline in a way that correlated with reduced ability of the original soil to suppress disease (Scher & Baker, 1980). The *Rhizoctonia* suppressive soil was found to contain the fungus *Trichoderma*, which alone was shown to be capable of transferring suppressiveness to conducive soil (Liu & Baker, 1980).

Studies such as those described above indicate the importance of bacteria in suppression of diseases, but offer no realistic approach to the application of the findings. Furthermore,

only a limited understanding of mechanism has been gained from such investigations. Given that bacteria are associated with suppression in the aforementioned experiments, considerable effort has been made to isolate and characterise specific antagonists with respect to their potential for biocontrol. These efforts allowed experiments in biocontrol to be conducted, revealing a number of important traits that contribute to effective biocontrol.

1.4 Bacteria in biocontrol.

As an initial screen for biocontrol candidates, large numbers of soil microbes have been isolated and screened for *in vitro* inhibition of pathogens. There has been considerable debate regarding the value of such screening given that a number of studies failed to find a correlation between *in vitro* inhibition and *in situ* disease suppression. However, some studies have successfully used *in vitro* screening to identify strains that show activity *in vitro* and *in vivo* (reviewed in Fravel, 1988). A number of strains have been isolated and characterised that show strong activity *in vitro* and *in situ*.

Bacteria representing several genera have been demonstrated to have the potential to control fungal or bacterial diseases. Among these are *Agrobacterium*, *Bacillus*, *Enterobacter*, *Erwinia*, and *Streptomyces* (Hadar et al., 1983; Kearns & Mahanty, 1998; Rothrock & Gottlieb, 1984; Ryder et al., 1999; Weller, 1988). Of increasing importance for biocontrol of soilborne pathogenic fungi are the fluorescent pseudomonads, which have shown great promise (Weller, 1988). A number have been isolated and examined in detail, in terms of their ability to control disease, and the mechanisms by which they act.

1.4.1 Experiments in biocontrol.

Given the lack of correlation between *in vitro* assays and disease suppression, it is important to demonstrate that the organisms in question have potential for use beyond laboratory-based studies. This is initially achieved using glasshouse and field trials. Such investigations have been carried out as a means to assess the potential of bacterial isolates for control, and also to establish whether host plants of differing disease susceptibility contribute to variability in biocontrol (King & Parke, 1993; Rankin & Paulitz, 1994; Ryder & Rovira, 1993).

1.4.2 *Pseudomonas spp.* as biocontrol agents.

Fluorescent *Pseudomonas* spp. are abundant in nature. Perhaps this is why they are so often isolated in the search for biocontrol agents. Many fluorescent pseudomonads have proved their worth in biocontrol by suppressing disease symptoms and enhancing crop productivity, and useful candidates are still being found (Ross et al., 2000). Coupled with the appeal of the variety of secondary metabolites they produce, many are vigourous colonisers of the rhizosphere, which is a desirable trait in the suppression of root disease. The observations of plant growth stimulation and root colonisation have led to a number of *Pseudomonas* spp. being grouped among the so-called "plant growth promoting rhizobacteria" (PGPR) (Weller, 1988). Experiments demonstrating the *in situ* efficacy of *Pseudomonas* spp. isolates in biocontrol are numerous. Examples of their successful application include the suppression of *Pythium ultimum* and *Rhizoctonia* on cotton seedlings, suppression of *Fusarium* wilt on radish by seed treatment, suppression of tobacco root rot, and enhanced yields of sugar beet (Howell & Stipanovic, 1979; Howell & Stipanovic, 1980; Keel et al., 1989; Leeman et al., 1995b; Suslow & Schroth, 1982). While these studies provide clear evidence for the potential application of biocontrol, they do not adequately examine the mechanism of control. The long-standing question of whether biocontrol efficacy can be explained by nutrient competition, antibiosis, induction of systemic resistance in the plant, or a combination of effects remains unanswered.

1.5 Mechanisms involved in biocontrol.

The ability of certain strains to act as biocontrol agents is probably a consequence of evolutionary adaptations that allow the organism to maintain its existence in complex and difficult environments. For this reason, it seems likely that the organisms that provide the best biocontrol would use a number of strategies to maximise their survival in competitive environments. Among these strategies are traits that have come to be viewed as important for the successful utilisation of organisms for biocontrol.

1.5.1 Competition for nutrients.

Fungal pathogens need nutrients for germination of spores and subsequent invasion of the host. Clearly, one mechanism that could contribute to biocontrol would be to limit the availability of such nutrients (Baker, 1968). Establishing large populations of bacteria on roots (before planting or from seed coating) would effectively reduce the nutrients available to other organisms such as pathogens. Pathogens might also be excluded from preferred sites of attack, which are present where root exudates are abundant, by a pre-existing bacterial population (Weller, 1988). It is believed that the versatility of pseudomonads make them ideal for competitive exclusion (Weller, 1988). Baker (1968) reviewed literature that suggests competition for carbon, nitrogen, and vitamins all have a role in pathogen suppression. One further example that has been thoroughly investigated is competition for iron, mediated by siderophore compounds.

Siderophore production.

Siderophores are microbial iron(III)-transport agents (Leong, 1986), and have been defined as "a low-molecular-weight (500-1000 daltons) virtually ferric-specific legand (*sic*), the biosynthesis of which is carefully regulated by iron and the function of which is to supply iron to the cell" (Neilands, 1981). This definition is sufficiently broad to accommodate a range of structures, while being specific in its functional requirements. A large number of microbes produce siderophores of some kind in response to low levels of extracellular iron. This is because although iron is an abundant element, it is generally found in the Fe^{3+} (ferric) state, which is largely insoluble in water, and thus inaccessible as a nutrient. At pH7, ferric iron is soluble in water at about 10^{-17}M, however microbes require concentrations around 10^{-6}M for growth (Leong, 1986; O'Sullivan & O'Gara, 1992). To circumvent the problem of iron availability, microbes manufacture and secrete siderophores to mediate high affinity iron transport. Uptake of the ferric-siderophore complex is via a siderophore-specific receptor, and the iron is likely to be released from the complex by reduction, since siderophores have a low affinity for Fe^{2+} (Leong, 1986). Soil pseudomonads often produce fluorescent siderophore compounds, which are classified as either pyoverdins or pseudobactins (O'Sullivan & O'Gara, 1992).

What role do siderophores play in biocontrol? The most obvious suggestion would be that by sequestering available ferric iron, siderophores make the ferric iron unavailable to all but the siderophore producer. Because of the specific nature of siderophore uptake, it is generally the case that only the producing organism can benefit. Experimental evidence comes from the isolation of the siderophore pseudobactin from *Pseudomonas sp.* B10. It was demonstrated that the pseudobactin from B10 was able to reduce fungal populations on the rhizoplane of potato much like the producing organism did, and enhance plant growth. This growth enhancement was not apparent when ferric-siderophore was applied, indicating the iron sequestering ability of the pseudobactin as the mechanism (Kloepper et al., 1980a). In disease control trials, addition of the pseudobactin to conducive soils was correlated with induced suppression of take-all of wheat and *Fusarium* wilt of flax (Kloepper et al., 1980b). Although some siderophores are potent antibiotics (Neilands, 1981), evidence of this nature would appear to eliminate an antibiotic effect since the only variable was the iron concentration. As long as completely pure siderophore is used, this kind of experiment has the potential to go beyond simply implicating siderophores in biocontrol, and generate data that could reveal a mechanism for the contribution siderophores make to biocontrol.

An alternative experimental approach, the use of siderophore-defective mutants, has yielded varied results. Although such experiments cannot specify a role for the siderophore, experiments with mutants are a powerful tool for testing the involvement of siderophores in biocontrol. For example, Paulitz and Loper (1991) generated pyoverdine (Pvd) mutants of *P. putida*, and demonstrated that these strains could suppress *Pythium* damping-off of cucumber as well as the wildtype, indicating no role for Pvd. The fact that the mutants were non-fluorescent suggested that the strain of *P. putida* did not produce additional fluorescent siderophore compounds (Paulitz & Loper, 1991). In contrast, fluorescent siderophores were shown to be important in the control of *Pythium ultimum* by a *P. fluorescens* strain. Mutants that no longer produced siderophore were less able to protect cotton from pre-emergence damping-off than the wildtype, although it is interesting to note that the mutants were more protective than untreated controls (Loper, 1988). This result suggests a significant contribution by a siderophore, and some additional control from an unidentified factor. Further experiments that utilise antibiotic defective, siderophore-producing strains derived from disease suppressing wildtypes, have also been

conducted. An example of this approach is the analysis of the role siderophores play in disease suppression by *P. fluorescens* 2-79, which is known to produce the antibiotic phenazine-1-carboxylic acid (PCA). PCA has previously been implicated in biocontrol processes using strain 2-79 (Thomashow & Weller, 1988), so the role of siderophores was assessed by comparing the ability of siderophore mutants, PCA mutants, and siderophore/PCA double mutants to suppress *G. graminis* var. *tritici* (Hamdan et al., 1991). In both PCA plus and minus backgrounds, the ability of 2-79 to produce fluorescent siderophores had little influence on disease suppression. The work outlined above, although not exhaustive, suggests the conclusion that siderophores can be, but are not always, important in biocontrol. One problem with assessing the role of siderophore-mediated iron binding mechanisms is that other mechanisms for iron binding are not excluded. Given the inhibitory nature of some siderophores, it is possible that their contribution is varied, depending on the specific compound involved. In mechanistic terms, the contribution of siderophores to biocontrol probably falls into place somewhere between rhizosphere competence and antibiosis.

1.5.2 Induced systemic resistance.

In addition to direct interactions with pathogens, some evidence exists suggesting that PGPR can indirectly cause suppression of fungal pathogens by inducing resistance in the host plant. Two examples of this have demonstrated suppression of foliar pathogens by PGPR (van Peer et al., 1991; Wei et al., 1991). In both examples, the use of foliar pathogens enabled an assessment of induced resistance without the complicating factor of direct interaction between pathogen and control agent. The PGPR could not be isolated at the sites of pathogen infection, suggesting that suppression was mediated by host plant resistance. Since resistance was increased when plant roots were already populated by PGPRs, the observed resistance was concluded to result from PGPR-induced resistance. It is important to note that not all disease suppressing PGPR can induce systemic resistance. Of 94 strains tested by Wei et al (1991), only six appeared to induce resistance, although all 94 were thought to be capable of suppressing *P. ultimum* or *R. solani*, or promoting seedling emergence (Wei et al., 1991). The situation with respect to root pathogens is somewhat more complex. Since it is difficult to physically separate pathogen and biocontrol agent, interactions and other suppression mechanisms may interfere with

assessment of induced resistance. Nonetheless, induced resistance to black root rot of tobacco has been suggested to be a contributing factor in the ability of *P. fluorescens* CHA0 to control the disease. In his review on biocontrol, Weller (1988) suggests that HCN can induce resistance to black root rot caused by *Thielaviopsis basicola*. HCN production has been shown to contribute to suppression of black root rot by *P. fluorescens* CHA0 by experiments in which a HCN mutant was less suppressive than the wildtype, and transfer of HCN production genes to a normally non-suppressing strain conferred the ability to control the disease (Voisard et al., 1989). In another set of experiments, the induction of systemic resistance to *Fusarium* wilt was found to be promoted by the O antigen side chain of *P. fluorescens* LPS (Leeman et al., 1995a). Using bioassays in rockwool growth cubes, Leeman et al (1995) were able to physically separate root pathogen and biocontrol agent. By using O antigen mutants and cell wall extracts from those mutants and the wildtype, the contribution of O antigen to *Fusarium* wilt resistance was examined. The wildtype strain and its purified LPS were able to suppress *Fusarium* wilt whereas the O antigen mutants and purified LPS from those mutants could not suppress the disease (Leeman et al., 1995a). Although these experiments cannot definitively eliminate other mechanisms, they provide compelling evidence to suggest that induced resistance can contribute to biocontrol in some cases.

1.5.3 Rhizosphere colonisation.

Successful colonisation of the root environment is critical to the biocontrol of root pathogens, since it is in the vicinity of the root that the pathogens operate (O'Sullivan & O'Gara, 1992). There are a number of factors that influence the ability of bacteria to successfully colonise the rhizosphere. These include features of the soil as well as important bacterial traits. As outlined previously, soil properties include pH, structural features (e.g. clay versus sandy), temperature, and mineral content, but experimentation to distinguish between these has been difficult (O'Sullivan & O'Gara, 1992). The presence of biological entities such as bacteriophage and protozoa can also influence colonisation, by killing the biocontrol organism, although at least one study suggests predation by protozoa is not a factor in rhizosphere competence (Jjemba & Alexander, 1999; Stephens et al., 1987). The colonisation strategies that bacteria have evolved are somewhat simpler to study, although an *in vivo* assay system is still required.

Cell surface polysaccharides, osmotolerance, pili, and flagella have all been implicated as important for successful root colonisation (Weller, 1988). Exopolysaccharide (EPS) could aid in bacterial adherence, since it binds cells together and may help reduce the likelihood of being displaced by indigenous microbes. Pili are thought to allow attachment, while flagella allow bacteria to actively move to desirable locations. This mechanism may work along with chemotaxis. If there is sufficient soil moisture, chemotaxis and motility would allow bacteria to move toward root exudates (Weller, 1988), while the contribution flagella could make in low moisture environments would be minimal. Although flagella-mediated motility has been implicated as important in some studies (de Weger et al., 1987; Schippers et al., 1987), other studies are contradictory (Bowers & Parke, 1993; Howie et al., 1987; Scher et al., 1988). Recently, molecular studies on *in vitro* biofilm formation by *Pseudomonas* spp. have implicated pili and flagella in this process (O'Toole & Kolter, 1998a; O'Toole & Kolter, 1998b). In a recent review, it is suggested that rhizosphere colonisation might be an example of biofilm formation (Davey & O'Toole, 2000). If indeed this proves to be the case, the use of *in vitro* biofilm mutants may give further insight into the role of flagella, pili and chemotaxis.

A *P. putida* isolate that has plant growth-promoting properties and disease suppression capabilities (*Fusarium* wilt of cucumber) was shown to interact with agglutinin, a plant protein that causes agglutination of the bacterium (Anderson, 1983). Agglutination appeared to play a role in colonisation, and mutants that could not be agglutinated were less able to colonise (Anderson et al., 1988). Interestingly, those mutants were also reduced in their ability to suppress disease and enhance plant growth (Tari & Anderson, 1988). These results show that agglutination can be an important factor in colonisation, and also correlate colonisation with biocontrol. However, the role agglutination plays in long term colonisation is less certain, and Glandorf et al (1994) concluded that agglutination may be important in short term colonisation, but their evidence did not support a decisive role in root colonisation for all *Pseudomonas* spp. (Glandorf et al., 1994).

In a recent study, the role of superoxide dismutase activity has been assessed in relation to rhizosphere colonisation (Kim et al., 2000). In that study, *P. putida* mutants were made

with mutations in either *sodA* (encodes manganese-superoxide dismutase), *sodB* (encodes iron-superoxide dismutase), or both *sodA* and *sodB*. These mutants were assessed for their ability to colonise the rhizosphere of bean plants, alone and in competition with the parental strain. Both the *sodA* and the *sodB* mutants were able to colonise as well as the wildtype, while the *sodA/sodB* mutant was impaired. In competition, the *sodA* mutant was sufficiently competitive to remain at a similar population to the wildtype, while the *sodB* mutant and the double mutant were out-competed by the wildtype. Two possibilities were offered as explanations. Firstly, the active metabolism at growing root tips could lead to high levels of reactive oxygen species to which *sod* mutants are sensitive. The alternative explanation was that increased O_2^- generation in the mutants could inhibit metabolic pathways, reducing the ability of the *sod* strains to metabolise available nutrients (Kim et al., 2000). Clearly this study is intriguing, but the significance in broad terms will be established when comparative studies with other PGPR are carried out.

In an innovative study of rhizosphere colonisation by *P. fluorescens* SBW25, Rainey (1999) used *in vivo* expression technology to trap promoters that were specifically induced in the rhizosphere environment. Rainey demonstrated that at least 20 genes were specifically induced in the rhizosphere, and suggested that these genes were important for successful colonisation (Rainey, 1999). Clearly, rhizosphere colonisation is a multifactorial process, and the current understanding of the process is incomplete.

1.5.4. Antibiotics involved in biocontrol.

Despite the numerous different bacterial strains that have been shown to have biocontrol potential, it seems there may be comparatively few antibiotics that are of importance in the suppression of fungal diseases. Among the well studied *Pseudomonas* spp., there is a recurring theme of disease control by the same small group of compounds. In addition to the contribution to disease control, these compounds have been studied for their role in ecological fitness, and molecular studies have uncovered the genes required for production and regulation of these compounds. The current understanding of the most well-characterised of these antimicrobial metabolites, phenazines, 2, 4-diacetylphloroglucinol, pyoluteorin, pyrrolnitrin and HCN is outlined below.

PHENAZINES.

Phenazine (Phz) antibiotics are produced by a number of fluorescent pseudomonads, and are derived from chorismate. There are over 50 different phenazine compounds known to occur in nature, and some organisms simultaneously produce multiple phenazines (Turner & Messenger, 1986). These molecules have broad activity against bacteria and fungi, and it is possible that DNA intercalating activity and/or oxidative activity of phenazines contribute to the broad spectrum of activity (Turner & Messenger, 1986).

Role in disease suppression.

The contribution of phenazine antibiotics to biocontrol of root diseases is well established. In experiments with *P. fluorescens* 2-79, comparisons of disease control provided by the wildtype and Phz mutants revealed that Phz mutants were deficient in suppression of take-all of wheat. The control could be restored by complementation of the mutation, supporting the importance of Phz biosynthesis in biocontrol (Thomashow & Weller, 1988). Further investigations with strain 2-79 showed that the presence of this strain resulted in few *G. graminis* var. *tritici* lesions on wheat roots, while Phz mutants could not prevent lesion formation (Bull et al., 1991). To more directly address the issue of Phz production and its correlation with disease control, Thomashow et al (1990) recovered the antibiotic from the roots of plants that grew from seeds treated with the Phz producers *P. fluorescens* 2-79 or *P. aureofaciens* 30-84. No antibiotic could be recovered from plants that were grown from seeds treated with Phz mutants, and the recovery of antibiotic correlated with lower levels of take-all (Thomashow et al., 1990). In an inventive approach to address the role of Phz in disease suppression, Mazzola et al (1995) examined the Phz sensitivity of various *G. graminis* strains and found Phz resistant varieties of *G. graminis*. Interestingly, Phz-producing strains were able to suppress disease caused by *G. graminis* strains that were sensitive to Phz *in vitro*, but these strains could not control disease caused by Phz-resistant *G. graminis* (Mazzola et al., 1995). This study revealed a useful correlation between Phz sensitivity *in vitro* and the control by Phz producing pseudomonads *in situ*.

Ecological role.

In addition to its usefulness in combating fungal diseases, examination of Phz production by the strains 2-79 and 30-84 has demonstrated a more fundamental role for the antibiotic.

It seems that the ability to produce Phz improves the rhizosphere competence of these bacteria. In long term studies, Phz-producing 2-79 and 30-84 survived better than Phz mutants in both bulk soil and rhizosphere environments. When tested in pasteurised soil, the antibiotic mutants were better able to maintain their population size. These results suggested that Phz production was important for survival in the presence of indigenous microflora, probably because the antimicrobial properties of Phz allow producing organisms to kill their competitors. Additionally, Phz seemed to be more important for survival on healthy roots than diseased, possibly due to the fact that take-all lesions lead to nutrient leakage from the plants, which allows the rhizosphere to sustain higher populations (Mazzola et al., 1992). Since biocontrol strains such as *P. aureofaciens* 30-84 interact with the target fungus on the rhizoplane (Pierson & Pierson, 1996), it is probably not surprising that the relative nutrient levels on the root surface are important factors for microbial competition in the rhizosphere. The experiments above indicate that the ability to produce Phz gives producing strains an advantage in a highly competitive environment.

Molecular biology of phenazine production.

Recently, the genes required for Phz production in 2-79 have been described (Mavrodi et al., 1998). Using transposon mutagenesis, the region of DNA required for Phz production was found to span approximately 6kb, and transcription in this region was shown to proceed in a single direction. Analysis of DNA sequence revealed the presence of seven open reading frames, *phzABCDEFG*. The construction of chromosomal mutants of *phzC*, *D*, *E*, and *G* demonstrated that these genes were essential for Phz production. Expression of the entire gene cluster in *E. coli* resulted in PCA (Phenazine-1-carboxylic acid) production, whereas expression of *phzCDEFG* led to low levels of PCA plus a mixture of heterocyclic, nitrogen containing compounds. On the basis of DNA sequence similarity, functions for some gene products were predicted. The products of genes *phzCDEFG* are involved in the synthesis of phenazine-1,6-dicarboxylic acid (the precursor to PCA) from chorismate (Figure 1.1). While no DNA sequences submitted to various nucleotide databases yet match *phzA* or *phzB*, the observations made regarding PCA production in *E. coli* led to the tentative suggestion that the respective products might stabilise a multienzyme complex for PCA production, increasing efficiency and specificity. Finally, the high degree of similarity between the gene cluster in 2-79 and

that of 30-84 suggested that the seven gene cluster is required for PCA production, and other genes encoded proteins that are involved with modifying PCA to produce the variety of Phz derivatives seen in some organisms (Mavrodi et al., 1998). This suggestion was supported in a subsequent report. Delaney et al (2001) identified a gene called *phzO* downstream of the *phz* cluster in 30-84, and demonstrated that its product was required for the conversion of PCA to 2-hydroxyphenazine-1-carboxylic acid. In the same report, it is also suggested that the product of a gene called *phzH* in *P. chloraphis* converted PCA to phenazine-1-carboxamide (Delaney et al., 2001).

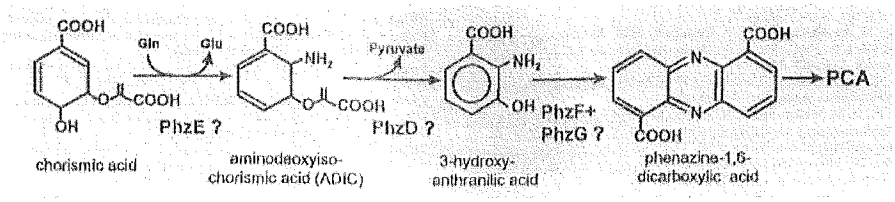


Figure 1.1. Proposed biosynthetic pathway for phenazine-1-carboxylic acid. Possible roles for PhzD, PhzE, PhzF, and PhzG are indicated. This figure was reproduced from Mavrodi et al (1998).

Regulation of phenazine biosynthesis.

In addition to biosynthetic genes, the *phz* locus also encodes the genes *phzR* and *phzI*, which are required for cell density-dependent regulation of PCA synthesis in *P. aureofaciens* 30-84 (Pierson et al., 1994; Wood & Pierson, 1996). The *phzR* gene was identified by screening cosmids for the ability to enhance expression of a *phzB*::*lacZ* fusion (Pierson et al., 1994), while *phzI* was found by DNA sequencing adjacent to *phzR* (Wood & Pierson, 1996). The *phzR/I* system positively regulates PCA expression, due to accumulation of a diffusible *N*-acyl-homoserine lactone molecule synthesised by PhzI, and subsequent activation of PhzR, which in turn leads to increased expression of *phz* genes (Pierson & Pierson, 1996).

2, 4-DIACETYLPHLOROGLUCINOL.

The antibiotic 2, 4-diacetylphloroglucinol (Phl) is produced via polyketide biosynthesis by a number of plant-associated *Pseudomonas* spp. (Bender et al., 1999). Strains that produce Phl have been shown to occur in a number of soils that suppress take-all of wheat

(Raaijmakers et al., 1997), and the biosynthetic locus for Phl production appears to be conserved among diverse Phl-producing pseudomonads (Keel et al., 1996).

Role in biocontrol.

The importance of Phl production for biocontrol of black root rot of tobacco has been established (Keel et al., 1990). A Phl-defective mutant of *P. fluorescens* CHA0 was shown to be significantly less able to suppress black root rot of tobacco than either the wildtype strain or an *in trans*-complemented Phl mutant. Additional indirect evidence for the role of Phl in biocontrol comes from a study by Raaijmakers et al (1997), in which a correlation emerged between soils suppressive to take-all of wheat, and Phl-producing bacteria. Of seven soils examined, the three suppressive soils harboured Phl-producing populations of between $5.1x10^5$ and $1.6x10^6$ cfu/g of root, while Phl-producers in the conducive soils were either undetectable or present at 40-fold lower numbers (Raaijmakers et al., 1997). While not formally showing the importance of Phl production in the suppressive soils, this correlation certainly adds support to the suggestion that Phl is important for biocontrol. The argument is further strengthened by the use of HPLC to demonstrate that Phl was produced on the roots of wheat grown in the suppressive soils, but could not be detected on wheat roots from conducive soils (Raaijmakers et al., 1999). It is important to note that although the presence of Phl-producing bacteria and Phl on roots correlates with suppression of disease, differences in soil composition could also have an influence on disease suppression.

Molecular biology of Phl production.

Two recent reports describe the molecular characterisation of the biosynthetic region for Phl. The initial work demonstrated that a 6.5kb region from *P. fluorescens* Q2-87 could confer Phl production upon normally non-producing *Pseudomonas* species, and mutagenesis of the region defined a 5kb sequence with two divergent transcriptional units (Bangera & Thomashow, 1996). Subsequently, a cluster of six genes for Phl biosynthesis was identified within the 6.5kb clone from Q2-87. These genes were designated *phlACBDEF* (Bangera & Thomashow, 1999). The DNA sequence analysis shows that *phlACBDE* are oriented in the opposite direction to *phlF*, as predicted in the earlier study by Bangera and Thomashow (1996). Bangera and Thomashow (1999) carried out

functional studies that suggested that PhlD was essential for the production of MAPG (monoacetylphloroglucinol, the precursor to 2,4-diacetylphloroglucinol), possibly by condensation of two acetoacetyl-CoA molecules, producing an eight-carbon polyketide, followed by a cyclisation reaction, yielding MAPG. These putative functions are supported by the sequence similarity of PhlD to CHS/STS genes from plants, which are homodimeric proteins capable of catalysis of condensation reactions and subsequent cyclisation in polyketide synthesis. The gene products PhlA, PhlB, and PhlC were suggested to function together, since mutations in any have the same phenotypic consequences. They are necessary for conversion of MAPG to 2, 4-DAPG, but also seem to be required for MAPG synthesis (Figure 1.2). In addition to the biosynthetic genes, a putative transport protein (PhlE) was identified, its gene located upstream of *phlACBD*, and a regulator gene (*phlF*) was found upstream of the other genes, encoded in a divergent direction.

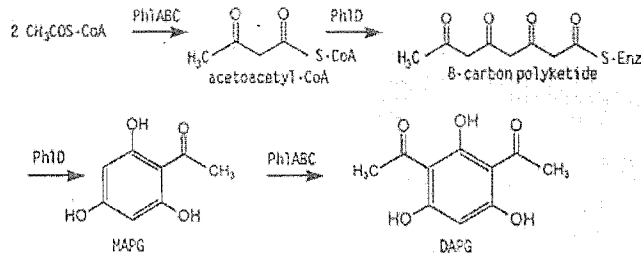


Figure 1.2. Biosynthetic scheme for 2,4-diacetylphloroglucinol. Possible roles for PhlA, PhlB, PhlC, and PhlD are shown. This figure was taken from Bangera and Thomashow (1999).

Regulation of Phl synthesis.

When the genes for Phl were identified, it was suggested that the gene *phlF* encoded a repressor protein, based upon a number of observations. Firstly, a clone of the Phl gene cluster lacking an intact *phlF* can confer Phl production upon normally non-producing *Pseudomonas* strains, but a larger clone which included *phlF* was unable to confer the ability to synthesise Phl (Bangera & Thomashow, 1996). In addition, Phl production is enhanced in *P. fluorescens* strains harbouring a chromosomal copy of *phlACBDE*, with a truncated *phlF* (Bangera & Thomashow, 1999). Thirdly, sequence similarity with other repressors, and the presence of a helix-turn-helix motif in the predicted translation product, is consistent with the identification of PhlF as a DNA-binding regulator protein (Bangera & Thomashow, 1999). The *phlF* gene has also been found in *P. fluorescens* F113 by Delany et al (2000), and its role as a repressor has been further substantiated by three observations

from that study. Firstly, purified PhlF was shown to bind specifically to the intergenic region between *phlF* and *phlA* (these genes are divergently transcribed). Secondly, over-expression of PhlF in F113 resulted in a greater than 90% reduction in Phl production. Finally, a *phlF* mutation in F113 led to significant increases in Phl synthesis. In contrast to the impact of *phlF* mutation and PhlF over-expression on Phl production, production of other secondary metabolites by F113 was unaffected, strongly supporting PhlF as a specific regulator of Phl (Delany et al., 2000). In *P. fluorescens* CHA0, *phlA* was shown to be autoinduced by Phl, and repressed by other secondary metabolites including pyoluteorin, and PhlF was concluded to mediate both processes (Schnider-Keel et al., 2000)

PYOLUTEORIN.

Pyoluteorin is a polyketide antibiotic composed of a resorcinol ring linked to a bichlorinated pyrrole, and is inhibitory toward bacteria and fungi (Bender et al., 1999).

Role in biocontrol.

P. fluorescens Pf-5 was analysed during an investigation into the biocontrol of *P. ultimum* damping-off disease on cotton seedlings. It was known that Pf-5 produced pyrrolnitrin, but this antibiotic was not inhibitory to *P. ultimum*. However, Pf-5 could still inhibit *P. ultimum* growth *in vitro*, suggesting production of a second antibiotic. This was subsequently shown to be pyoluteorin. In the course of their study, Howell and Stipanovic (1980) showed that treating cotton seeds with Pf-5 or purified pyoluteorin led to improved survival in *P. ultimum*-infested soil, indicating a role for pyoluteorin in disease suppression (Howell & Stipanovic, 1980). Contrary to this observation, Kraus and Loper (1992) showed little correlation between the ability of Pf-5 to produce pyoluteorin and the suppression of pre-emergence damping off in cucumber (caused by *P. ultimum*). Mutants defective in pyoluteorin biosynthesis were not significantly worse than the wildtype strain at protecting the cucumbers (Kraus & Loper, 1992). Thus, the role of pyoluteorin in biocontrol has not been unequivocally demonstrated, and the possibility that different host and pathogen systems might respond to pyoluteorin production must be considered.

Molecular biology of pyoluteorin synthesis.

A number of studies have led to a working model for pyoluteorin biosynthesis. The genomic region of Pf-5 that encodes pyoluteorin was first identified by mutagenesis, and subsequently isolated by Kraus and Loper (Kraus & Loper, 1992; Kraus & Loper, 1995). In their 1995 study, Kraus and Loper demonstrated that mutations causing a pyoluteorin-defective phenotype spanned nearly 21kb. Using fusions to the promoterless ice nucleation gene (*inaZ*) it was shown that the genes within the pyoluteorin region were likely to be transcribed in a single direction from at least two promoters (Kraus & Loper, 1995). Further investigations revealed that there were ten open reading frames within the pyoluteorin biosynthetic region (Nowak-Thompson et al., 1999), and that the largest two of these (*pltB* and *pltC*) appear to encode proteins with sequence and organisational similarity to type I polyketide synthases (PKSs) (Nowak-Thompson et al., 1997). Nowak-Thompson et al (1997) proposed that the putative PKSs could synthesise the resorcinol moiety that constitutes part of pyoluteorin. A pathway based upon the ten ORFs that make up the pyoluteorin cluster has been proposed (Bender et al., 1999), and is outlined briefly here (see Figure 1.3). The polyketide synthases PltB and PltC use an activated precursor in the synthesis of the resorcinol ring. Such a precursor is likely to be proline, which could be activated by PltF (a putative acyl-CoA-synthetase), since starter units are often activated as acyl-CoA derivatives. PltG is proposed to be responsible for terminating the assembly of the polyketide because it has similarity to thioesterases. Synthesis of the pyrrole ring is thought to be carried out by PltE, a putative acyl-CoA dehydrogenase. The proteins PltA, PltD, and PltM all have similarity to halogenases, so are proposed to be involved in the chlorination of the pyrrole ring (Bender et al., 1999). PltL has no known function, and PltR is a LysR-type transcriptional regulator (Nowak-Thompson et al., 1999).

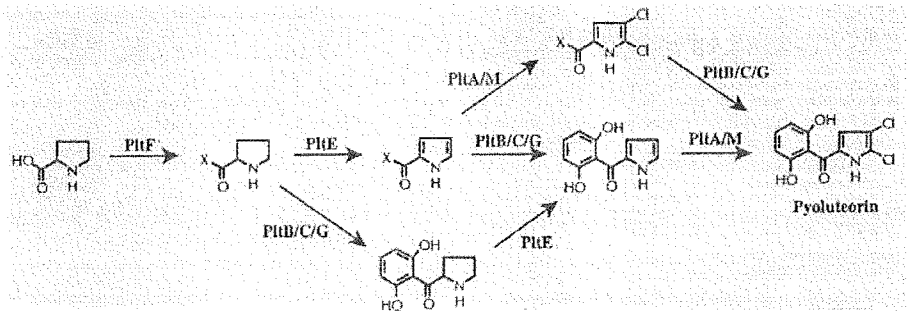


Figure 1.3. Model for biosynthesis of pyoluteorin. Two alternative variations are shown, and the possible roles for the proteins Plt, PltA, PltB, PltC, PltE, PltF, PltG, and PltM are suggested. This scheme was taken from Nowak-Thompson et al (1999).

Regulation of pyoluteorin biosynthesis.

In the study of the pyoluteorin biosynthetic cluster, the gene *pltR* was sequenced and found to encode a LysR-type transcriptional regulator. Mutational studies demonstrated that PltR was necessary for pyoluteorin production. Transcriptional fusion studies showed that PltR was required for the expression of pyoluteorin biosynthetic genes *pltB*, *pltE*, and *pltF*, suggesting a specific role for PltR in the regulation of pyoluteorin biosynthesis (Nowak-Thompson et al., 1999).

PYRROLNITRIN.

Pyrrolnitrin is an antifungal metabolite produced by a number of *Pseudomonas* spp, and tryptophan is the precursor for its synthesis (Kirner et al., 1998). In addition to *Pseudomonas* spp, an *Enterobacter agglomerans* strain has also been shown to produce pyrrolnitrin, and purified pyrrolnitrin from this strain exhibited broad spectrum antifungal and antibacterial activity (Chernin et al., 1996).

Role in biocontrol.

As with the other antibiotics detailed above, experiments have revealed that pyrrolnitrin is important in the biocontrol activity of producing strains. In a study analogous to early investigations into pyoluteorin, it was shown that treatment of cotton seeds with a *P. fluorescens* isolate, or the pyrrolnitrin it produced, resulted in increased protection of cotton seedlings from *R. solani* (Howell & Stipanovic, 1979). An investigation into suppression of *Fusarium sambucinum* dry rot suggested a correlation between suppression by *P. cepacia* B37w and pyrrolnitrin production (Burkhead et al., 1994). The strength of this study was that pyrrolnitrin could be isolated from the site of fungal infection, indicating the biocontrol agent produced the compound *in situ*. However, although not detected, production of other antifungal compounds was not ruled out. A more conclusive investigation was carried out by Hill et al (1994). Mutants of *P. fluorescens* BL915 unable to produce pyrrolnitrin had a concomitant loss of biocontrol efficacy. Furthermore, a genetic locus isolated from wildtype BL915 was able to confer pyrrolnitrin production and disease suppression upon normally non-producing *P. fluorescens* strains (Hill et al., 1994). In contrast, a study using pyrrolnitrin mutants of *P. cepacia* J82 failed to find a correlation between antibiotic production and disease suppression (McLoughlin et al., 1992). In their

experiments on sunflower wilt fungus (*Sclerotinia sclerotiorum*), McLoughlin et al showed that pyrrolnitrin-defective mutants suppressed disease as effectively as the wildtype. Thus, it may be that disease suppression by pyrrolnitrin production is system- or environment-specific, and therefore not effective in every situation.

Molecular biology of pyrrolnitrin biosynthesis.

Using mutants of *P. fluorescens* BL915, Hill et al (1994) was able to isolate a genomic region that could not only restore pyrrolnitrin production to the mutants, but was capable of conferring pyrrolnitrin production upon other *P. fluorescens* strains (BL914 and BL922). These results indicated that the genomic clone was genetically sufficient for pyrrolnitrin production, and subcloning suggested that the required genes were found within a 4.5kb region (Hill et al., 1994). However, subsequent analysis revealed that the BL915 fragment encoded the global regulator GacA, and the heterologous hosts were genetically capable of pyrrolnitrin biosynthesis, but lacked the required positive regulator. In order to isolate biosynthetic genes, Hammer et al (1997) took advantage of BL914. Genes in BL914 that required GacA for expression were identified by using a transposon with a promoterless *lacZY* as a promoter-probe. Fusions to *lacZY* that were activated upon introduction of *gacA/S* were studied further, since these represented mutants with insertions in GacA-dependent genes. One such mutant could not produce pyrrolnitrin, but did produce other GacA regulated products. The genomic region flanking the transposon insertion was used as a probe to isolate a cosmid with the wildtype sequence from BL915. Mutagenesis was used to show that the genes for pyrrolnitrin were located within a 6.2kb region, and DNA sequencing identified four open reading frames (*prnABCD*), probably transcribed as one unit. A clone of these four genes under control of the *tac* promoter could confer pyrrolnitrin production upon *E. coli*, supporting the suggestion that the four genes encode the requirements for pyrrolnitrin biosynthesis (Hammer et al., 1997). The functions of the products of each gene were elucidated using deletion mutants of each, and examination of the intermediate products that accumulated (Kirner et al., 1998) (see Figure 1.4). PrnA converts tryptophan to 7-chlorotryptophan, which is then used by PrnB to synthesise monodechloroaminopyrrolnitrin (MDA). MDA is converted to aminopyrrolnitrin (APRN) by PrnC, and finally PrnD oxidises the amino group of APRN to a nitro group, thus completing the production of pyrrolnitrin (Kirner et al., 1998). Comparison with other

pyrrolnitrin-producing strains indicated that there is a high degree of conservation among pyrrolnitrin biosynthetic loci (Hammer et al., 1999).

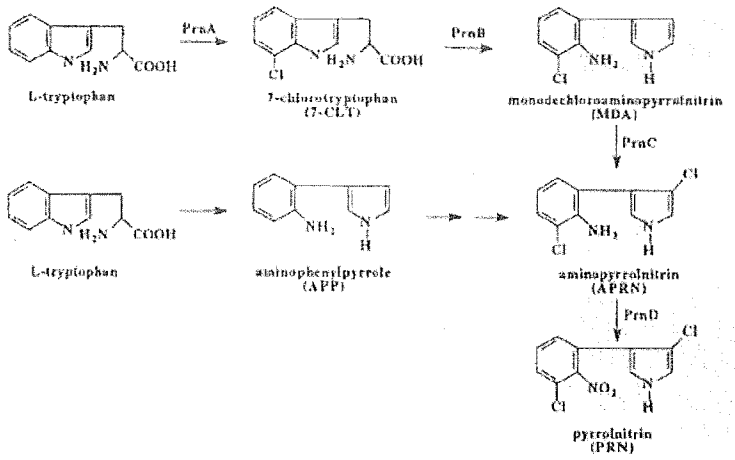


Figure 1.4. Biosynthetic pathway for pyrrolnitrin biosynthesis. The steps catalysed by PrnA, PrnB, PrnC, and PrnD are indicated. This figure was reproduced from Kirner et al (1998). The lower pathway is the previously accepted model for pyrrolnitrin biosynthesis.

HYDROGEN CYANIDE.

Although HCN has been implicated in the induction of systemic resistance, its role in biocontrol could be more complex given the toxic nature of the compound. The fact that HCN can inhibit the *in vitro* growth of phytopathogenic fungi is worth considering in the context of an antimicrobial compound, although inhibition of fungi in the rhizosphere has not been shown (Blumer & Haas, 2000a).

Biosynthesis of HCN.

A genetic locus encoding HCN biosynthesis in *P. fluorescens* CHA0 was first isolated by Voisard et al (1989). They found that an 8.4kb region of CHA0 genome was capable of conferring HCN production upon another *P. fluorescens* strain. Disruption of a 5kb subclone with an antibiotic resistance cassette, and subsequent introduction of this mutation into the CHA0 genome resulted in loss of HCN synthesis (Voisard et al., 1989). In a subsequent study, the genes involved in HCN synthesis were located by mutagenesis, and found to occupy a 3.8kb region of the CHA0 genome. Nucleotide sequence data indicated the presence of three open reading frames, labelled *hcnABC* (Laville et al., 1998).

Expression of these genes, under control of the T7 promoter in *E. coli*, led to HCN production. This confirmed their role in HCN synthesis. The products HcnA, HcnB, and HcnC are probably subunits of the HCN synthase. The predicted amino acid sequence of each subunit shows similarity with known dehydrogenases, supporting the dehydrogenase model for HCN synthesis. This model suggests that glycine is oxidised to iminoacetic acid, and the C-C bond is split in a second dehydrogenase reaction, releasing HCN and CO_2 (Laville et al., 1998). This reaction is shown in Figure 1.5.

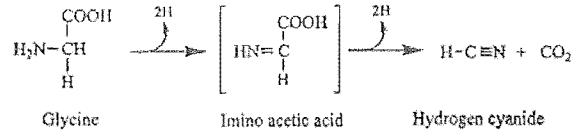


Figure 1.5. Production of HCN by HCN synthase (comprised of HcnABC). This reaction scheme was taken from Blumer and Haas (2000).

Regulation of HCN biosynthesis.

Production of HCN in *P. fluorescens* CHA0 is stimulated by glycine, the biosynthetic precursor, probably via a post-translational mechanism (Blumer & Haas, 2000a). In addition, HCN production in *P. fluorescens* CHA0 is regulated by ANR (anaerobic regulator of arginine deaminase and nitrate reductase) (Laville et al., 1998). The role for ANR was deduced from the observation that the –40 region of the promoter for *hcnA* had a consensus FNR/ANR binding sequence, and the fact that HCN is produced under oxygen-limited conditions. Subsequent mutagenesis of *anr* yielded a strain that produced very little HCN, and could not express an *hcnA*::*lacZ* fusion, but could still produce PHL and pyoluteorin (Laville et al., 1998).

METABOLITE SPECIFIC AND GLOBAL REGULATION.

Of the antifungal metabolites discussed above, all but pyrrolnitrin have been associated with specific mechanisms for their regulation. Due to the fact that the regulator genes are often found in close proximity to the target genes, the genes responsible for the regulation can often be identified during DNA sequencing of the biosynthetic region encoding a particular antibiotic. This appears to have been the case for Phl and pyoluteorin regulation, but not for the identification of regulators for HCN production by *P. fluorescens* CHA0, or

phenazine production by *P. aureofaciens* 30-84. In addition to the specific regulation of these metabolites, each is also under the influence of at least one global regulatory mechanism. Indeed, in *P. fluorescens* CHA0, the two-component system GacA/GacS controls HCN, Phl, and pyoluteorin (Laville et al., 1992). The global regulation of antifungal metabolites is discussed in chapter 5.

1.5.5 Nutritional influences on antibiotic production.

Since antibiotic production appears to be one of the most important traits for biocontrol, an assessment of environmental factors influencing antibiotic production is essential. One variable feature that could impact upon biocontrol performance is the type and availability of elemental nutrients, such as minerals and carbon source. A number of studies have approached this topic, mostly using liquid culture conditions to examine the impact of various nutrients on antibiotic production. Production of three antibiotics by *P. fluorescens* HV37a was shown to vary depending upon glucose concentration in solid media. One antibiotic required a minimum glucose concentration (0.2%), whereas expression of the other two antibiotics was optimal with no added glucose (James & Gutterson, 1986). In a study of PCA production by *P. fluorescens* 2-79, adenine reduced production while cytosine increased PCA accumulation. The minerals $FeSO_4$, H_3BO_3, $MgSO_4$, and $ZnSO_4$ all appeared to enhance PCA production when added to cultures with low inoculum density, but the increase in PCA correlated with increased growth, indicating that the effect of these minerals on PCA production probably related to improved growth. However, when added to a culture with a high inoculum density, $FeSO_4$, $ZnSO_4$, and $(NH_4)_6Mo_7O_{24}$ led to increased PCA accumulation in a manner that was not growth dependent, and adding both zinc and iron led to a further increase of PCA (Slininger & Jackson, 1992). In a more comprehensive study, Duffy and Défago (1999) examined the effect of minerals and carbon source on Phl, pyoluteorin, and pyrrolnitrin biosynthesis by *P. fluorescens* CHA0. Eleven different minerals were added to NBY medium (nutrient broth and yeast extract), either alone or with glucose or glycerol supplementation. $ZnSO_4$, $(NH_4)_6Mo_7O_{24}$, glycerol and glucose all increased Phl production. In addition, a synergistic effect was observed with glycerol plus each of $ZnSO_4$, $(NH_4)_6Mo_7O_{24}$, and $CuSO_4$. No significant interactions between mineral treatment and glucose were reported. Pyoluteorin production was enhanced by $ZnSO_4$, $CoCl_2$, and glycerol. Glycerol plus $CoCl_2$ or $ZnSO_4$ increased

pyoluteorin compared to the control with no minerals, but these combinations produced less antibiotic than the mineral-alone cultures. Fructose, mannitol and glycerol significantly increased both Phl and pyoluteorin production in the presence of $ZnSO_4$ and $(NH_4)_6Mo_7O_{24}$. Pyrrolnitrin production was increased by fructose, mannitol, glycerol and glucose. The effect of zinc, inorganic phosphate, and glucose was then assessed on a range of biocontrol strains that had been grouped into three groups based upon amplified ribosomal DNA restriction analysis (ARDRA). The effect of $ZnSO_4$ on Phl production was significant in ARDRA group 1, which comprised 11 of the 42 strains, but only influenced two other strains. Phl production was stimulated by glucose in all but six strains. Pyoluteorin was only made by ARDRA group 1, and while its production by most strains was stimulated by $ZnSO_4$, it was reduced below the detection limit in glucose enriched media. Inorganic phosphate at greater than 10mM reduced Phl and pyoluteorin synthesis by all strains tested, but did not affect pyrrolnitrin by CHA0 (Duffy & Defago, 1999). In a glasshouse trial using gnotobiotic systems, Fe^{3+} was shown to be important in disease suppression, and this ability correlated with the stimulation of HCN production by Fe^{3+} in the test strains (Keel et al., 1989).

It is evident from the research outlined above that biocontrol of fungal root pathogens using fluorescent Pseudomonads is influenced by a myriad of factors. Not only must the organism be capable of establishing itself in the rhizosphere, it must have a means by which to suppress the pathogen. In the majority of cases it seems as though producing one or more antifungal metabolite enables pathogen suppression, but other possibilities such as siderophore production and induction of systemic resistance may also be important, either alone or in combination with antibiosis. However, even if the organism in question possesses the desirable traits, efforts in biocontrol can be thwarted by environmental factors such as nutrient availability, predation, and soil structure. Biocontrol has unquestionable promise, either as a replacement for chemical pesticides or as part of an integrated management system, but there is clearly considerable research still to be done.

1.6 *P. aureofaciens* PA147-2.

P. aureofaciens PA147 was isolated from soil at Lincoln, near Christchurch, New Zealand during a search for soil organisms with biocontrol potential, and was identified on the basis

of API 20 NE strip analysis. Subsequently, a spontaneous rifampicin resistant mutant was isolated and designated PA147-2 (Carruthers, 1994). PA147-2 is a Gram-negative rod with a single polar flagellum (Figure 1.6), and recent analysis of the 16s rDNA sequence showed it is most closely related to a *P. aureofaciens* type strain (S. Godfrey, Pers. Comm.). PA147-2 was chosen for study as a potential biocontrol agent because of its broad spectrum *in vitro* antifungal activity (Carruthers, 1994).

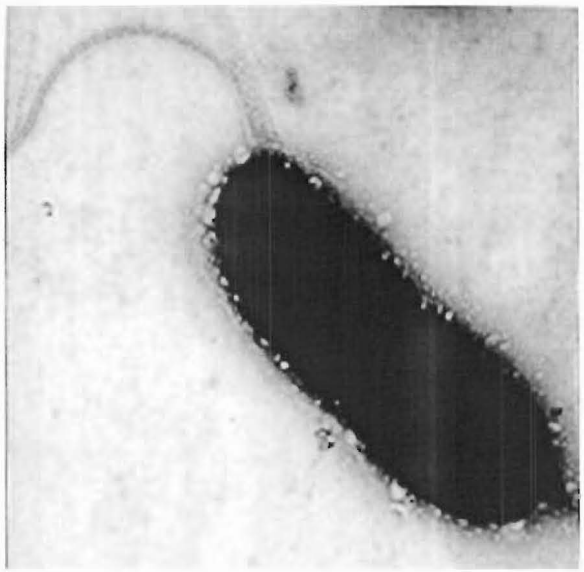


Figure 1.6. Transmission electron micrograph of *P. aureofaciens* PA147-2. The single polar flagellum is clearly visible.

1.6.1 Previous research on fungal inhibition by PA147-2.

Molecular biology.

In an effort to understand the molecular basis of antifungal activity from PA147-2, Carruthers (1994) used Tn*5* to generate a collection of mutants that were deficient in their *in vitro* antifungal activity (Af⁻). The DNA flanking the transposon insertion points of three mutants was sequenced. Two of these mutants were auxotrophs – PAF30 required phenylalanine, tyrosine and tryptophan for growth, while PAF35 needed to be supplied with arginine and uracil. The DNA sequence from PAF30 indicated a mutation in *aroB*, the gene encoding 3-dehydroquinate synthase, which is required early in the shikimic acid pathway. The insertion in PAF35 was found to be in *carA*, the gene for the small subunit of carbamoylphosphate synthetase, which is an intermediate in the biosynthesis of both

arginine and uracil. Thus, the nucleotide sequence was in agreement with the auxotrophic requirements of these two mutants (Carruthers, 1994). The third mutant to be analysed was PA109. The transposon was found to be in a 16kb *Eco*RI fragment from PA147-2, and preliminary sequence data indicated it interrupted the gene for a two-component regulator. However, the sequence was not completed by Carruthers. Analysis of the 16kb region using saturation mutagenesis indicated the presence of other genes that were required for antifungal activity, and these were presumed to be regulated by the two-component regulator (Carruthers, 1994). Allele replacement of the mutation in PA109 by a cosmid from the PA147-2 genomic library (creating PA109R-186) was shown to restore antifungal activity (Carruthers et al., 1994).

Disease suppression.

Although PA147-2 is a strong inhibitor of fungal growth *in vitro*, it was important to establish its ability to suppress disease *in planta*. A glasshouse trial was conducted in which PA147-2, PA109 and the allele replacement strain PA109R-186 were assessed for their ability to suppress *Phytophthora* rot of asparagus (Carruthers, 1994; Carruthers et al., 1995). The results indicated that PA147-2 and the PA109R-186 were able to suppress disease, which correlated with *in vitro* antibiosis. PA109 was unable to suppress *Phytophthora* rot. Interestingly, although PA109 did not significantly reduce root rot, it did reduce the incidence of diseased shoots, and all strains tested appeared able to enhance the growth of asparagus seedlings (root length and plant weight) independent of the presence of the fungal pathogen. These data supported the suggestion that PA147-2 was a good biocontrol candidate, and indicated it could provide additional benefits to plants (Carruthers, 1994; Carruthers et al., 1995).

Biochemical analysis.

Two researchers have carried out experiments aimed at identifying the chemical nature of the antifungal compound(s) produced by PA147-2. Initially, crude antifungal extracts were prepared from PA147-2 grown on solid media. A bacteria/agar slurry was extracted with 80% acetone, and after acetone removal the aqueous fraction was applied to a C8 column. An active fraction was eluted with methanol and subjected to HPLC analysis using a 0.1% trifluoroacetic acid-acetonitrile gradient (30-60%) over 20 minutes. Comparison of HPLC

traces for PA147-2 and three mutants (PA109, PA1, and PA138) showed that all three mutants were missing a peak, and antifungal assays demonstrated that the peak correlated with antifungal activity. Unfortunately insufficient sample was prepared for further purification (Carruthers, 1994). In a more recent study, Godfrey (1997) attempted the further characterisation of antifungal compounds from PA147-2. Initially it was demonstrated that PA147-2 does not produce HCN or PCA. Further analysis consisted of preparing a crude extract from plate cultures, and separating compounds by polarity using dry flash chromatography. Two fractions, one polar and the other non-polar, showed activity, and were further investigated. Circular chromatography was used to separate the non-polar extract into three fractions, one of which showed activity. However, mass spectrometry analysis suggested that the active fraction actually contained three compounds that were thought to be decomposition products from the active compound. The active polar fraction from dry flash was examined using C18 reverse-phase HPLC. While separation was achieved, yields were either too low or not stable enough for a positive antifungal assay result. The conclusion was that at least one compound in the extracts from PA147-2 was relatively unstable, and hence difficult to chemically characterise, and obtaining sufficient compound for detailed analysis was extremely difficult (Godfrey, 1997).

1.7 Objectives of the present study.

The present study was undertaken with two broad objectives. Firstly, to increase the understanding of the molecular basis of antifungal activity by PA147-2, using a previously created collection of mutants. A second objective was to complement the molecular studies with biological experiments that relate to the biocontrol potential of PA147-2. To address the broad objectives, several aims were defined at the beginning and during the research. These are outlined below.

1. Create a system that would allow *trans* complementation experiments to be carried out.
2. Broadly characterise all mutants in the collection on a molecular level. The outcome of this aim was intended to determine subsequent goals.
3. Characterise mutants PA109, PA138, and PAH26 with respect to the impact the Tn*5* insertion has on antifungal activity.

4. Conduct a field trial to further establish the potential of PA147-2 as a biocontrol agent.
5. Based upon observations of bacterial survival in the field, carry out preliminary experiments to examine the fitness of PA147-2, and the possibility that producing antifungal compounds has a negative impact on fitness.

Biochemical characterisation of the antifungal compound(s) produced by PA147-2 was not a direct objective of this research for two reasons. Firstly, work by Godfrey (1997) indicated that the compound lacked stability in extracts, making analysis extremely difficult. Secondly, the mutants that were analysed by DNA sequencing in the preliminary study did not have disruptions in any gene known to encode a protein with a direct role in antibiotic synthesis. An investigation into the identity of the antifungal compound(s) is ongoing in collaboration with the department of chemistry, University of Canterbury.

Chapter 2

Experimental Procedures

2.1 Bacterial strains, plasmids and phage.

All bacterial strains and phage used in this study are listed in Table 2.1. Plasmids not created in this study are also listed in Table 2.1. Plasmids constructed during this investigation are described in detail in section 2.7.

Table 2.1. Bacterial strains, plasmids and bacteriophage

Strain, plasmid or phage	Genotype/Description	Reference or source
E. coli		
PB2480	*thr*1 *leu*6 *lacY*1 *supE*44 *tonAZ*1	P. Berquist
CSR603	*phr*1 *recA*1 *uvrA*6 *thr*1 *leuB*6 *argE*3 *thi*1 *ara*14 *lacY*1 *galK*2 *xyl*5 *mtl*1 *supE*44 *tsx*33 *gyrA*96 *rpsL*31	(Sancar et al., 1979)
DE880	Δ(*lac-argF*)*U169 relA1 thi-1 cps-3 sulA::*Mu d (*lac* Ap)XCam (Mu+) *srlC300*::Tn*10*	(Ennis et al., 1989)
DE1491	Δ(*lac-argF*)*U169 relA1 thi-1 cps-3 malF55::*Tn*5 sulA::*Mu d (*lac* Ap)XCam (Mu+) Δ(*recA-srlR*)301::Tn*10*	(Ennis et al., 1989)
DH5α	*supE44* Δ*lacU169*(ϕ*lacZ*ΔM15) *hsdR17 thi-1 relA1 recA1 endA1 gyrA96*	(Hanahan, 1983)
ZK4	*araD139* Δ(*lacIPOZYA-grgF*)*U169 rpsL thi recA56 supO*	(Gilson et al., 1987)
MT2	*ilv his rpsL recA1* (N7N53cI+)	(Toman et al., 1985)
S17-1	*thi pro hsdR- hsdM*$^+$ Δ*recA* RP4-2-Tc::Mu-Km::Tn*7*	(Simon et al., 1983)
W3110	Wild-type K12	(Kohara et al., 1987)

Table 2.1. Continued.

Strain, plasmid or phage	Genotype/Description	Reference or source
P. aureofaciens		
PA147-2	Rf^R wildtype; antifungal (Af) positive	(Carruthers et al., 1994)
PA109	Tn*5* mutant of PA147-2; Rf^R, Km^R; Af$^-$	"
PAH26	Tn*5* mutant of PA147-2; Rf^R, Km^R; Af$^-$, biofilm defective	(Carruthers, 1994)
PA138	Tn*5* mutant of PA147-2; Rf^R, Km^R; Af$^-$	"
PA1	Tn*5* mutant of PA147-2; Rf^R, Km^R; Af$^-$	"
PAB108	miniTn*5* mutant of PA147-2; Rf^R, Km^R; Af$^-$, Biofilm defective	(Monds, 2000)
PAE21	Tn*5* mutant of PA147-2; Rf^R, Km^R; Af$^-$	(Carruthers, 1994)
PA168	Tn*5* mutant of PA147-2; Rf^R, Km^R; Af$^-$	"
PAF30	Tn*5* mutant of PA147-2; Rf^R, Km^R; Af$^-$	"
PAF35	Tn*5* mutant of PA147-2; Rf^R, Km^R; Af$^-$	"
PAI17	Tn*5* mutant of PA147-2; Rf^R, Km^R; Af$^-$	"
PAI75	Tn*5* mutant of PA147-2; Rf^R, Km^R; Af$^-$	"
PAI95	Tn*5* mutant of PA147-2; Rf^R, Km^R; Af$^-$	"
PA138R9	PA138 allele replacement strain; Af$^+$	This study
PA138R16	PA138 allele replacement strain; Af$^+$	"
PA147-2*finR*Δ	PA147-2 with *finR* deletion; Rf^R, Km^R; Af$^-$	"
PA147-2*finA606*	PA147-2 with Gm^R insertion in *finA*; Rf^R, Gm^R; Af reduced	"
PA147-2*recA1*	PA147-2 with *recA*::Tn*10* 1; Rf^R, Km^R	"
PA147-2*recA4*	PA147-2 with *recA*::Tn*10* 4; Rf^R, Km^R	"
PA147-2*rec*Δ	PA147-2 with *recA* deletion; Rf^R, Km^R Gm^R	"
PA109*rec*Δ	PA109 with *recA* deletion; Rf^R, Km^R Gm^R	"
PA138*rec*Δ	PA138 with *recA* deletion; Rf^R, Km^R Gm^R	"
PAH26*rec*Δ	PAH26 with *recA* deletion; Rf^R, Km^R Gm^R	"
PA147-9.2	PA147-2 with mutation from pAF9.2 introduced into the genome by allele exchange; Rf^R, Km^R, Af$^+$	"
PA147-9.38	As for PA147-9.2, except created with pAF9.38; Rf^R, Km^R, Af$^+$	"

Table 2.1. Continued.

Strain, plasmid or phage	Genotype/Description	Reference or source
PA147-9.45	As for PA147-9.2, except created with pAF9.45; Rf^R, Km^R, Af^+	This study
PA147-9.55	As for PA147-9.2, except created with pAF9.55; Rf^R, Km^R, Af^+	"
PA147-9.6	As for PA147-9.2, except created with pAF9.6; Rf^R, Km^R, Af^+	"
PA147-9.66	As for PA147-9.2, except created with pAF9.66; Rf^R, Km^R, Af^+	"
PA147-9.68	As for PA147-9.2, except created with pAF9.68; Rf^R, Km^R, Af^+	"
P. aeruginosa		
PA01	Wildtype, clinical origin	(Holloway et al., 1977)
NT1	PA01 Δ*pstCAB-phoU*, Km^R	(Nikata et al., 1996)
Plasmids		
pACYC184	p15a *ori*; Cm^R, Tc^R	(Chang & Cohen, 1978)
pBLUESCRIPT (M13-, KS, SK)	ColE1 *ori lacZ*α/KS polylinker (polylinker in opposite direction for SK); Ap^R	Stratagene
pBR322	ColE1 *ori* Ap^R, Tc^R	(Bolivar et al., 1977)
pHP45-ΩCm	Ap^R, carries Ω Cm^R cassette	(Prentki & Krisch, 1984)
pHRP309	Gm^R, *lacZ'*, IncQ	(Parales & Harwood, 1993)
pHRP315	Ap^R, carries Ω Sm^R cassette flanked by multicloning sites	"
pHRP317	Km^R, carries Ω Sm^R cassette flanked by multicloning sites	"
pJQ200	Gm^R, has *sacB* gene for sucrose sensitivity	(Quandt & Hynes, 1993)
pLAFR3	pRK290 derivative; IncP-1; λ*cos*, pUC9 multicloning site and *lacZ*α; Tc^R	(Staskawicz et al., 1987)

Table 2.1. Continued.

Strain, plasmid or phage	Genotype/Description	Reference or source
pME6000	pBBR1MCS derivative; *lacZα*/multicloning site polylinker; mob; rep; Tc^R	Stephen Heeb
pME6001	pBBR1MCS derivative; *lacZα*/multicloning site polylinker; mob; rep; Gm^R	"
pUC18	ColE1 *ori*; *lacZα*/polylinker; Ap^R	(Yanisch-Perron et al., 1985)
pNK2859	plasmid containing miniTn*10* derivative 103	(Kleckner et al., 1991)
pPS132	pLAFR3 containing PA147-2 genomic DNA spanning the insertion point in PA138	Lab Collection
pPS192	pLAFR3 containing PA147-2 genomic DNA spanning the insertion point in PA138	"
pPS2122	pLAFR3 containing PA147-2 genomic DNA spanning the insertion point in PA138	"
pPS7138	Cosmid complementary to mutated region in PA109	(Carruthers et al., 1994)
pFC1	16kb::Tn*5* *Eco*RI clone from PA1 in pBR322; Ap^R, Tc^R, Km^R	"
pFC109	16kb::Tn*5* *Eco*RI clone from PA109 in pBR322; Ap^R, Tc^R, Km^R	"
pWT109	wildtype 16kb *Eco*RI region complementary to mutated region in PA109, cloned from pPS7138 in pACYC184; Tc^R	"
pREC1	Cosmid from PA147-2 genomic library. Confers MMS^R and UV^R on *recA* strains of *E. coli*; Tc^R, $recA^+$	(Silby & Mahanty, 2000)
Phage		
λNK1205	Lambda delivery vehicle for miniTn*10* derivative 112 (Km^R, *lacZ'*)	(Kleckner et al., 1991)
λNK1316	Lambda delivery vehicle for miniTn*10* derivative 103 (Km^R)	"
$P1_{vir}$		(Silhavy et al., 1984)

2.2 Fungal strains.

Fungal strains used in this study are listed in Table 2.2.

Table 2.2. Fungal strains

Fungus	Source[a]
Gaeumannomyces graminis var. *tritici*	Tony Cole[b]
Phytophthora megasperma var. *sojae*	ICMP 9489
Rhizoctonia solani	ICMP 11620

[a]ICMP = International collection of microorganisms from plants (Landcare research, Auckland, New Zealand). [b]Tony Cole, Department of Plant and Microbial Sciences, University of Canterbury.

2.3 Buffers, solutions and media.

Buffers, solutions and media used in this study were prepared as outlined in Appendix 1.

2.4 Bacteriological and fungal methods.

2.4.1 Routine culture conditions for bacteria and fungi.

All *E. coli* and *P. aureofaciens* strains were grown aerobically in LB (Sambrook et al., 1989), 1xA (Miller, 1972) or Pseudomonas minimal medium (PMM) (Kirner et al., 1996) at either 37°C or 30°C respectively unless otherwise specified. When required, media were solidified by the addition of 1.5% bactoagar (Gibco). Bacterial cultures were inoculated from a single colony from a selective plate or from frozen stocks. When required, antibiotics and supplements were added to culture media as outlined in Table 2.3. Fungal strains were grown on potato dextrose agar (PDA, Oxoid) at 21°C. Fresh plates were inoculated by removing a plug from the periphery of a growing colony (or from storage) and placing it at the center of a fresh plate.

Table 2.3. Antibiotics and supplements used in this study

Antibiotic or supplement	Final concentration
Ampicillin (Ap)	100 μg/mL
Chloramphenicol (Cm)	30 μg/mL
Cycloheximide	100 μg/ml
Gentamicin (Gm)	15 μg/mL
Kanamycin (Km)	50 μg/mL
Nalidixic acid (Nal)	30 μg/mL
Rifampicin (Rf)	50 μg/mL
Streptomycin (Sm)	50 μg/mL
Tetracycline (Tc)	15 μg/mL
Methyl methanesulphonate (MMS)	200 μg/mL
Mitomycin C (MC)	0.5 μg/mL
5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (Xgal)	20 μg/mL
5-bromo-4-chloro-3-indolyl-phosphate (BCIP)	20 μg/mL

2.4.2 Storage of bacteria and fungi.

For long term storage, bacterial strains were grown for 16 hours in LB, harvested by centrifugation, and frozen at –80°C in LB supplemented with 15% glycerol. For day to day use, streaks of bacteria were kept on agar plates at 4°C. For long term fungal storage, two methods were used: (i) plugs of fresh mycelium were taken from a fungal colony and frozen in 10% glycerol at -80°C; (ii) the fungal strains were grown on PDA slopes in universal bottles, then overlayed with sterile paraffin oil.

2.4.3 Collecting bacteria by centrifugation.

Bacteria were harvested from liquid culture and suspensions by centrifugation at 5000 *x g* unless otherwise specified. Centrifugation of bacteria was generally carried out at ambient temperature, except in the case of competent cell preparation, during which all centrifuge steps were conducted at 4°C.

2.4.4 Antifungal bioassay (Modified from Carruthers et al, 1994).

The *in vitro* assay for antifungal activity was carried out on Tris-buffered potato dextrose agar plates (TPDA). Tris-HCl (pH 7.6) was used at a final concentration of 80mM. TPDA plates were inoculated with the desired fungus and incubated for one to two days before bacteria for bioassay were streaked 1.5-2cm from the edge of the growing fungal colony. The bioassays were carried out at 21°C, and typically took between four and eight days before a definitive result could be seen.

2.4.5 Mutant crossfeeding assays.

Each of the Tn*5*-generated antifungal mutants of PA147-2 was grown in LB broth for 18 hours. As a control, aliquots (5μL) of each mutant were placed onto bioassay plates approximately 2cm from the growing fungal colonies, to confirm the mutant phenotypes. For the cross-feeding, aliquots of each mutant were combined with aliquots of each of the other mutants and then placed on bioassay plates for the inhibition assay. PA147-2 was included on each plate. Typically, eight combinations could be assessed on each bioassay plate. Each experiment was carried out three times to ensure reproducibility.

2.5 Conjugation, transformation and transduction.

2.5.1 Conjugation.

Plasmid DNA was routinely introduced to *P. aureofaciens* strains by conjugation. For all conjugation experiments, *E. coli* S17-1 was transformed by the appropriate plasmid in order to serve as the donor strain. The donor and recipient strains were grown overnight (approximately 18 hours) with relevant antibiotic selection and then cells from 500μL of culture were harvested by centrifugation. Cell pellets were washed twice with fresh LB, and finally resuspended in 200μL of LB. At this point the donor and recipient strains were mixed and an 100μL aliquot was pipetted onto a LB plate and allowed to dry. Plates were incubated at 30°C for between eight and 18 hours before scraping the bacteria from the surface and suspending them in LB. The suspension was spread plated onto LB agar plus antibiotics and returned to the incubator until colonies appeared.

2.5.2 Transformation.

CALCIUM CHLORIDE TRANSFORMATION OF *E. COLI.*

Calcium chloride transformation was carried out as described by Sambrook et al. (1989), with modifications. To prepare competent cells, the desired *E. coli* strain was grown to saturation in LB, subcultured (1:50) in fresh LB, and grown with vigorous shaking (300rpm) to an OD_{600} of 0.4-0.5. After cooling the culture on ice, the cells were collected, suspended in ½ volume of cold 10mM NaCl, and held on ice for five minutes. The cells were collected again and resuspended in ¼ volume of 100mM $CaCl_2$. After incubating on ice for ten minutes the bacteria were harvested, suspended in ⅛ volume of 100mM $CaCl_2$ and returned to ice for a further 30 minutes. In each transformation, 100-500ng of DNA was added to 50µL of competent cells, and this mixture was incubated on ice for 30 minutes. After the ice incubation the DNA/bacteria mix was subjected to a heatshock (42°C, 90 seconds) and then placed back on ice for two minutes. One mL of LB was added and the transformation mixture was incubated at 37° for one to two hours to allow expression of antibiotic resistance genes. Transformants were selected by plating aliquots of the transformation on LB supplemented with selective antibiotics. In cases where transformants were expected to be rare, the bacteria were concentrated by centrifugation, the supernatant was removed and the bacteria were resuspended in a smaller volume. This allowed the entire transformation mix to be plated.

TRANSFORMATION OF *E. COLI* BY ELECTROPORATION.

A 100mL exponential culture was started by diluting (1:100) a saturated broth culture of the desired *E. coli* strain in fresh LB. The culture was grown with vigorous shaking (300 rpm) to an OD_{600} of 0.6-0.8, after which it was placed on ice to cool. Bacteria were harvested, resuspended in 100mL of cold sterile dH_2O (sdH_2O), collected again and resuspended in 50mL sdH_2O. The cells were collected, resuspended in 1mL sterile 10% glycerol, centrifuged and finally resuspended in 500µL of 10% glycerol. Aliquots of cells (40µL each) were either used immediately or stored at -80°C until required. Stored competent cells were thawed on ice before use.

Electroporation was carried out with a Biorad Gene Pulser™ and Pulse Controller following the manufacturer's recommendations (Gene Pulser™ set at a capacitance of 25μFD and Pulse Controller set at 200Ω). Sterile electroporation cuvettes (0.1cm gap) and the chamber slide were cooled on ice prior to use. Approximately 100-200ng of DNA (dissolved in sdH_2O) was added to the competent cells, mixed and incubated on ice for one minute. The mixture was transferred to a cuvette, which was placed into the chamber slide in the electroporation chamber. The sample was pulsed at 18kV/cm. Immediately after the pulse 1mL of SOC (appendix 1) was added to the cuvette, and the entire sample was removed and placed into an Eppendorf tube and incubated at 37°C for one hour. Transformants were selected as described above for $CaCl_2$ transformation.

2.5.3 P1 Transduction.

PREPARATION OF P1 STOCK.

E. coli W3110 was grown in 3mL of LB broth for 18 hours before the addition of 25μL of 1M $CaCl_2$. The culture was returned to the 37°C shaking waterbath for ten minutes. A 1mL aliquot was transferred to an Eppendorf tube to which 100μL of $P1_{vir}$ stock was added. The phage were allowed to adsorb (without shaking) for ten minutes at 37°C prior to the addition of 10μL of 1M $CaCl_2$ and 20μL of 20% glucose. This mixture was added to 3mL of molten (50°C) H-Top agar (appendix 1) which was then poured onto a pre-warmed LB plate. After setting, the plate was incubated for eight hours at 37°C after which 5mL of cold LB broth was poured over the surface. The plate was then incubated at 4°C for 15 hours. The phage lysate was pipetted from the plate into a centrifuge tube. A 50μL aliquot of chloroform was mixed with the lysate prior to centrifugation (five minutes, 10000 *x g*, ambient temperature). The lysate was transferred to a sterile Universal bottle for use. The lysate was stored at 4°C in a Universal bottle with 25μL of chloroform.

P1 TRANSDUCTION.

P1 transduction was carried out essentially as described by Miller (1972). One mL of saturated recipient culture was harvested by centrifugation, resuspended in 1mL of MC buffer (appendix 1), and incubated at 37°C for ten minutes. Aliquots of 100μL were

transferred to three Eppendorf tubes, to which either 0, 10 or 100μL of $P1_{vir}$ stock was added. The phage were adsorbed at 37°C for 20 minutes and 100μL of 1M sodium citrate was added. The volume was increased to 1mL with MC buffer, and an aliquot (10μL) was removed for viable cfu/mL determination. The cells from the remaining 990μL were collected by centrifugation and plated on the appropriate medium (also containing 10mM sodium citrate) for growth of transduced bacteria.

2.6 DNA isolation and manipulation.

2.6.1 Isolation of plasmid DNA.

SMALL SCALE ALKALINE LYSIS. (MODIFIED FROM SAMBROOK ET AL, 1989)

A 500μl-1mL aliquot of a saturated bacterial culture was centrifuged in an Eppendorf tube to collect the cells. After removal of the supernatant by aspiration, the pellet was suspended in 100μL of solution 1. A 200μL aliquot of solution 2 was then added, and mixed until viscous and clear. One hundred and fifty microlitres of solution 3 was added and mixed well to break up the precipitate. The precipitate was removed from solution by centrifugation (ten minutes, 17000 *x g*, ambient temperature), and the supernatant was transferred to a clean tube. DNA was precipitated from the solution by the addition of 1mL of 100% ethanol, and the DNA was collected by centrifugation (ten minutes, 17000 *x g*, ambient temperature). The supernatant was removed by aspiration and the DNA pellet rinsed with 70% ethanol. The ethanol was removed by aspiration and the DNA was dried under vacuum. The pellet was dissolved in 30-100μL $T_{10}E_1$ (TE, appendix 1) or sdH_2O. This method generally yielded DNA of sufficient quality and quantity for analysis by restriction digestion and agarose gel electrophoresis. For larger yields and higher quality DNA the method below was used.

LARGE SCALE PREPARATION OF HIGH QUALITY DNA.

This method is a modification of the above alkaline lysis protocol. In this method the same first three solutions are used, in the same ratios employed above (for a 1mL bacterial sample). After removal of the precipitate produced by solution three, steps are taken to further purify the DNA by removing RNA and contaminating proteins. Outlined below is

the method for a 10mL culture, but this can easily be scaled up for higher yields from larger cultures.

The bacteria from 10mL of culture were collected in a 30mL centrifuge tube, and suspended in 1mL of solution 1. This was held on ice for five minutes prior to the addition of 2mL of solution 2. After mixing, the preparation was again held on ice for five minutes. Solution 3 (1.5mL) was then added and mixed. After five minutes of incubation on ice the precipitate was removed by centrifugation (ten minutes, 12000 *x g*, ambient temperature). The supernatant was transferred to a clean tube and the DNA was precipitated by the addition of 4.5mL of cold 2-propanol. After mixing well, the precipitate was recovered by centrifugation (ten minutes, 12000 *x g*, ambient temperature). The pellet was dissolved in 1mL sdH_2O, and then 1mL of 5M LiCl was added (Sambrook et al., 1989). The solution was thoroughly mixed, and placed on ice for ten minutes. The high molecular weight RNA precipitated by the addition of LiCl was removed from solution by centrifugation (ten minutes, 12000 *x g*, 4°C) and the supernatant was transferred to another clean tube. The DNA was precipitated with 2-propanol (2mL) and the resulting pellet dissolved in 200µL TE. The DNA solution was transferred to an Eppendorf tube and treated with RNase (final concentration 0.1µg/µL) for 15 minutes at 37°C. Contaminating proteins were removed by phenol/chloroform extraction (Section 2.6.3). The DNA was precipitated by adding 1/10 volume 3M sodium acetate and 2 volumes of 100% ethanol. DNA was recovered by centrifugation (ten minutes, 17000 *x g*, 4°C), washed with 70% ethanol, vacuum dried, and dissolved in 50-200µL TE or sdH_2O.

2.6.2 Preparation of genomic DNA.

Genomic DNA was prepared from *P. aureofaciens* strains using a rapid extraction with guanidium thiocyanate (Pitcher et al., 1989). The cells from 1mL of culture (grown for 16-20 hours) were collected in an Eppendorf tube by centrifugation and suspended in 100µL of TE prior to the addition of 500µL of GES lysis solution (appendix 1). This was incubated at 65°C for 15 minutes, followed by the addition of 250µL of cold 7.5M ammonium acetate. After ten minutes on ice, 500µL of 24:1 chloroform:isoamyl alcohol was added and mixed thoroughly by vortexing until an emulsion formed. The mixture was centrifuged

(ten minutes, 17000 *x g*, ambient temperature) and the top phase transferred to a new tube. The chloroform:isoamyl alcohol extraction step was repeated, and the top phase was transferred into a clean tube. To precipitate the DNA, 0.54 volumes of cold 2-propanol were added, mixed well, and held at ambient temperature for 10-15 minutes. At this stage the DNA could easily be seen floating in solution. The DNA was collected by centrifugation (two minutes, 17000 *x g*, ambient temperature), and washed three times with 70% ethanol as described for precipitation of DNA from solution (section 2.6.4). The DNA was dried under vacuum, and dissolved in 75μL TE.

2.6.3 Phenol/chloroform extraction of contaminants from DNA solutions.

When required, contaminants were removed from DNA solutions by phenol/chloroform extractions according to the method of Sambrook et al. (1989). Briefly, one volume of phenol saturated with Tris-HCl, pH 8 (Gibco BRL) and one volume of chloroform:isoamyl alcohol (24:1) were added to the DNA solution. The solutions were mixed until a homogenous emulsion formed, and centrifuged (five minutes, 17000 *x g*, ambient temperature). The aqueous (upper) phase was transferred to a new tube, and two volumes of 24:1 chloroform:isoamyl alcohol was added. The solutions were mixed and centrifuged, and the aqueous phase recovered. DNA was precipitated from solution using ethanol (section 2.6.4) and dissolved in an appropriate volume of TE or sdH_2O.

2.6.4 Precipitation of DNA from solution.

DNA was precipitated from solution as described by Sambrook et al. (1989) unless otherwise stated. A 0.1 volume aliquot of 3M sodium acetate was added to the DNA solution, followed by two volumes of cold (-20°C) 100% ethanol. After mixing, the DNA-containing tube was placed on ice for ten to 30 minutes, and the DNA was recovered by centrifugation (ten minutes, 17000 *x g*, 4°C). After removal of the supernatant, the DNA pellet was washed by the addition of 1mL of 70% ethanol and centrifugation for two minutes. The supernatant was again removed by aspiration, the DNA pellet dried under vacuum, and finally dissolved in an appropriate volume of TE or sdH_2O. DNA solutions were stored at 4°C or -20°C.

PRECIPITATION OF LIGATED DNA FOR ELECTROPORATION.

Prior to transformation of *E. coli* by electroporation, ligated DNA was precipitated. The ligation reaction volume was increased to 50μL by the addition of water. Potassium acetate (6.25μL of 2M, pH 8) was added, followed by 120μL of 100% ethanol. After mixing and incubation on ice (30 minutes), the DNA was recovered by centrifugation and washed with 70% ethanol as described above. The DNA was dissolved in 10μL of sdH_2O.

2.6.5 Restriction digestion of DNA in solution.

Digestion of DNA by restriction enzymes (Gibco BRL) was carried out according to manufacturer's instructions. Generally, digests were set up in 10-20μL volumes, and incubated at the appropriate temperature for one to four hours. If the restricted DNA was to be examined by electrophoresis, it was mixed with loading buffer (Section 2.6.7) and used. If the DNA was required for other purposes such as ligation, restriction enzymes were removed by phenol/chloroform extraction or inactivated by heat treatment (75°C, ten minutes).

2.6.6 Isolation, digestion and FIGE of total DNA in agarose plugs.

EMBEDDING BACTERIA IN AGAROSE.

Appropriate bacterial strains were grown for 16-18 hours in LB plus antibiotics. One mL aliquots were centrifuged, and the pellet resuspended in 1mL SE (appendix 1) buffer. The bacteria were centrifuged again, and resuspended in 1mL SE buffer for a second time. The bacterial suspension was incubated at 37°C for ten minutes to increase the temperature of the suspension, then 100μL of bacteria (approximately 1×10^8 cells) was mixed with 100μL of molten 2% agarose (dissolved in sdH_2O) which had been equilibrated to 50°C. The agarose/bacteria mixture was immediately pipetted into a plug mold (Biorad).

LYSIS OF CELLS EMBEDDED IN AGAROSE.

Once set, agarose plugs of bacteria were removed from the mold and incubated for 18 hours in 1mL lysis buffer (appendix 1). After lysis, plugs were carefully subjected to three, one hour washes with TE. Following the washes, plugs were stored at 4°C in TE.

RESTRICTION DIGESTION OF AGAROSE EMBEDDED DNA.

For each digest, ¼ of a plug was used. The plug was twice equilibrated in 400µL of 1x restriction buffer for 15 minutes each time. The plug was then placed in a clean tube with 200µL of 1x restriction buffer plus 10 units of *Spe*I and incubated at 37°C for 16-20 hours. One mL of TE was added to stop the reaction prior to electrophoresis.

FIELD INVERSION GEL ELECTROPHORESIS (FIGE).

Field inversion gel electrophoresis (FIGE) was used to separate and visualise the large DNA fragments produced by digesting agarose-embedded DNA with the rare-cutting restriction enzyme *Spe*I. FIGE was carried out by using a Biorad FIGE Mapper™ Electrophoresis System according to the manufacturer's recommendations.

A 1% gel was made by dissolving 1g of agarose in 100mL of 0.5x TBE. This was used to cast a 5mm thick, 15 well gel in a 15cm x 15cm casting tray. While the gel was left to cool, the digested agarose-embedded DNA samples were equilibrated in TBE by first being placed in 1mL of 0.5 x TBE, incubated at ambient temperature for 15 minutes, then transferred to 1mL of fresh 0.5 x TBE and equilibrated a further 15 minutes at ambient temperature. The samples were loaded into the wells of the gel with a spatula, pushed to the front of the wells, and sealed in with agarose (the same solution used to cast the gel).

Approximately 1.6L of 0.5 x TBE was poured into the FIGE Mapper electrophoresis cell and circulated at approximately 0.75L/minute by using the variable speed pump. The casting tray and gel were placed into the electrophoresis cell, and a buffer coverage of 2mm was achieved by adding or removing buffer. Run parameters were determined empirically, and are described in appendix 2. Electrophoresis was carried out at ambient temperature for the appropriate time. A Biorad pulsed-field standard lambda concatemer was used as a molecular weight standard. The gel was stained and processed as described below (section 2.11.1)

2.6.7 Agarose gel electrophoresis of DNA.

Agarose gel electrophoresis was used to separate and visualise DNA fragments from restriction digests and PCRs. Agarose was dissolved in 1x TAE (appendix 1) to give a final concentration of 0.6-1.5%, depending on the size of DNA fragments to be visualised. Gels were cast with either 8, 12, or 16 wells using casting apparatus supplied with the Hoefer HE33 mini horizontal submarine unit. Once the gel was prepared, an appropriate volume of 6x loading buffer (appendix 1) was added to the DNA samples, which were then loaded into the gel. Electrophoresis was carried out in a Hoefer HE33 mini horizontal submarine unit, typically at 7V/cm with 1x TAE as the running buffer. Gels were stained in a 0.5μg/mL solution of ethidium bromide. DNA was visualised on an Ultralum UV transilluminator (254nm) and an image was produced by using a Kodak Digital Science Electrophoresis Documentation and Analysis System (EDAS) 120. Sizes of DNA fragments were determined by comparison to known standards. Molecular weight standards were the 100bp ladder (Biorad), the 1kb ladder (Biorad) and bacteriophage lambda DNA digested with *Hin*dIII (Gibco BRL).

2.6.8 Dephosphorylation of 5' single stranded DNA.

When linearised plasmid vectors had compatible ends, 5' phosphate groups were removed to prevent vector self-ligation. The dephosphorylation was accomplished using Roche Calf Intestinal Alkaline Phosphatase (CIAP). The vector DNA was recovered from the restriction digest by ethanol precipitation, and dissolved in sdH_2O. Typically dephosphorylation reactions were carried out in 50μL volumes containing 5μL 10 x CIAP buffer, CIAP (for each μg of DNA, one unit of CIAP was used), DNA and sdH_2O. The reaction was incubated at 37°C for one hour and the CIAP was inactivated by heating to 75°C for ten minutes, or removed by phenol/chloroform extraction. After ethanol precipitation the vector was used in ligations.

2.6.9 DNA ligation.

COHESIVE END LIGATION.

Generally, ligation reactions between DNA molecules with compatible cohesive termini were carried out in 20μL volumes consisting of 4μL of 5x DNA ligase buffer, 1μL of DNA

ligase (Life Technologies) and 100-500ng of DNA. An insert to vector ratio of approximately 3:1 was used. The reaction was allowed to proceed at 20°C for one to 16 hours. The DNA was ethanol precipitated prior to use in electrotransformation of *E. coli.*

BLUNT END LIGATION.

To ligate DNA molecules with blunt ends 20μL reactions were used. These were as described for cohesive-end ligation except that 2.5μL of DNA ligase was used, and approximately 1μg of DNA was included in the reaction. Blunt end ligations were incubated at 14-16°C for 20 hours. As for cohesive-end ligations, the DNA was ethanol precipitated before further use.

2.6.10 Filling in 5' overhangs.

To create blunt ends on restriction fragments that had 5' single stranded DNA overhangs, the ends were filled in using the 5'-3' polymerase activity of the Klenow fragment of DNA polymerase 1 (Life Technologies), as described (Ausubel et al., 1989). DNA was digested as outlined above (section 2.6.5) and either treated with phenol/chloroform (section 2.6.3), recovered from an agarose gel (section 2.6.11) or used directly. If the DNA was used directly, the reaction volume was increased to 20μL by the addition of 1μL of appropriate restriction buffer, 2.5μL of 0.2mM dNTPs, 2 units of the Klenow fragment and sdH_2O. This reaction was incubated at 30°C for 15 minutes before being stopped by the addition of 1μL 0.5M EDTA and heating to 75°C for ten minutes. The DNA was purified by phenol/chloroform extraction. For other DNA samples, a 20μL reaction mixture consisting of DNA, 2.5μL dNTPs, 2μL of Klenow reaction buffer, two units of the Klenow fragment and sdH_2O was made. This was treated as outlined above.

2.6.11 Recovery of DNA from agarose gels.

When required, fragments of DNA were extracted from agarose gels using the Prep-A-Gene® DNA purification kit (Biorad) following the manufacturer's instructions. The desired DNA fragment in an ethidium bromide-stained gel was visualised on a Sigma T2210 UV transilluminator (302nm), excised using a scalpel, and placed in an Eppendorf

tube. The volume of gel slice was estimated by assuming 1g of gel slice is equivalent to 1mL. Based on the volume of the gel slice and the desired amount of Prep-A-Gene matrix to be added (typically 5-10μL), three volumes of Prep-A-Gene binding buffer was added to the gel slice. This was incubated at 50°C until the gel slice had dissolved (about ten minutes). Prep-A-Gene matrix was added (5μL per μg of DNA to be recovered) and incubated at ambient temperature for ten minutes with frequent agitation. The matrix (with bound DNA) was removed from solution by centrifugation (one minute, 17000 xg, ambient temperature) and then resuspended in a volume of DNA binding buffer equivalent to 25 times the volume of matrix used. The DNA-bound matrix was centrifuged again, and resuspended in 25 times the matrix volume of 80% ethanol. After repeat centrifugation, the DNA-bound matrix was suspended in the same amount of 80% ethanol and re-centrifuged. The pellet was allowed to air-dry before resuspending in two pellet volumes of sdH_2O and incubating at 37°C for 15 minutes to allow DNA to be eluted from the matrix. The mixture of DNA solution and matrix was separated by centrifugation and transfer of the supernatant to a clean tube. This step was repeated to ensure no residual matrix inadvertently remained in the DNA solution.

2.7 Plasmid constructs.

2.7.1 Cloning Tn*5* insertion points.

A series of clones was made to allow the DNA sequence flanking the Tn*5* insertion point in mutants to be ascertained. The majority of these clones took advantage of the unique *Sal*I site in Tn*5*, which is found at base 2684, and the relative frequency of *Sal*I sites in the *P. aureofaciens* genome. These features allowed the recovery of Km^R clones that had the 5' 2684bp of Tn*5* (encodes Km^R) plus adjacent *P. aureofaciens* DNA. A number of insertion points also were cloned with *Eco*RI which has no recognition site in Tn*5*, resulting in Km^R clones that contain an intact Tn*5* flanked by *P. aureofaciens* DNA on both sides (see Figure 2.1 for illustration).

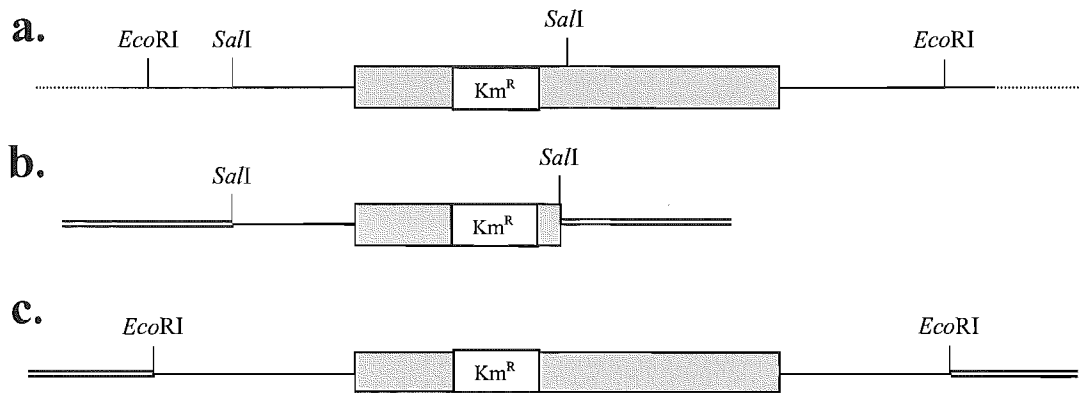


Figure 2.1. Strategy for cloning Tn*5*-interrupted sequences. Grey boxes indicate Tn*5*. **a.** Diagram of Tn*5* inserted into the genome of PA147-2. Broken lines indicate continuation of the genome in both directions. **b.** *Sal*I clone derived from the Tn*5* mutated region. Note that the clone has Tn*5* sequence remaining (~2.7kb), which includes the Km^R gene. This clone only contains chromosomal DNA on one side of the Tn*5*. Double lines indicate cloning vector DNA. **c.** *Eco*RI clone derived from the Tn*5* mutated region. This clone contains the entire Tn*5*, and has chromosomal DNA on each side of the transposon. Double lines indicate vector DNA.

pCM1.

4kb *Sal*I fragment from PA1 cloned in pBLUESCRIPT KS-. Immediately adjacent to the transposon are the first 43 bases of *finT*.

pCM109.

5kb *Sal*I fragment from PA109 cloned in pBLUESCRIPT KS-. The clone contains the 5' 1371 bases of *finT*.

pCM138.

4kb *Sal*I fragment from PA138 cloned in pBLUESCRIPT KS-. The cloned sequence contains *finR* interrupted by Tn*5*.

pCM168.

6.4kb *Sal*I fragment from PA168 cloned in pBLUESCRIPT KS-. DNA sequence adjacent to the transposon has no significant similarity to known genes.

pCME21.

6.5kb *Sal*I fragment from PAE21 cloned in pBLUESCRIPT KS-. The Tn*5* is within a sequence showing similarity to a galactosyl transferase gene.

pCMH26.
4.2kb *Sal*I fragment from PAH26 cloned in pBLUESCRIPT KS-. *pstA* is interrupted by the transposon in this clone.

pCMI17.
5.8kb *Sal*I fragment from PAI17 cloned in pBLUESCRIPT KS-. DNA sequence adjacent to the transposon has no significant similarity to known genes.

pCMI75.
7kb *Sal*I fragment from PAI75 cloned in pBLUESCRIPT KS-. The transposon interrupts a sequence with similarity to the 3' end of an alginate lyase gene.

pCMI95.
6.9kb *Sal*I fragment from PAI95 cloned in pBLUESCRIPT KS-. The predicted translation product derived from the sequence adjacent to the transposon has similarity to RagC and other multidrug efflux pumps.

pCM138E.
11.5kb *Eco*RI fragment from PA138 cloned in pBLUESCRIPT KS-. This clone contains *finR* with a Tn*5* insertion.

pCMA3E.
20kb *Eco*RI fragment from PAA3 cloned in pBLUESCRIPT KS-. The DNA sequence flanking the transposon in this clone has no significant similarity to known genes.

pCMA3B.
9kb *Bam*HI-*Eco*RI fragment from pCMA3E cloned with pME6000.

pCMH26E.
9.5kb *Eco*RI fragment from PAH26 cloned in pBLUESCRIPT KS-. This clone contains *pstA* with a Tn*5* insertion, *pstC*, and part of *pstS*.

2.7.2 *recA* constructs.

pPRAM1 AND pPRAM4.
The plasmids pPRAM1 and pPRAM4 were created by insertional mutagenesis of pREC1 with miniTn*10* derivative 112 (section 2.15). Both plasmids could confer Tc^R and Km^R

upon host bacteria, but were unable to confer UV and MMS resistance. pPRAM1 has a miniTn*10* insertion approximately 500 bp into *recA*, while pPRAM4 has an insertion approximately 950 bases into the *recA* gene.

pPRAM1.1 AND pPRAM4.1.

These plasmids were made by digesting pPRAM1 and pPRAM4 with *Bam*HI and ligating with *Bam*HI digested pBR322. The desired clones were selected on the basis of Km^R, the gene for which is not interrupted by the *Bam*HI site in the miniTn*10*. The resulting subclones have approximately half of the miniTn*10* (one copy of IS10) and PA147-2 DNA flanking the insertion point in the cosmid. pPRAM1.1 has approximately 200 bases of the PA147-2 *recA* gene, while pPRAM4.1 has approximately 600 bases of the *recA* gene.

pREC6G.

Subcloning of an MMS^R region from pREC1 was accomplished by digesting the cosmid with *Eco*RI and *Hin*dIII, and ligating to identically digested pME6001. The ligation was used to transform *E. coli* DH5α to Gm^R and MMS^R. A 6kb *Eco*RI/*Hin*dIII fragment was found to confer MMS (methane methyl sulphonate) resistance, and the clone was designated pREC6g.

pREC6M1, pREC6M2, AND pREC6M3.

pREC6g was mutagenised using miniTn*10* derivative 103 (Km^R). Restriction mapping demonstrated that each of these clones has a different insertion, as shown in Figure 2.2. Each of these clones is Gm^R and Km^R, and unable to confer MMS^R upon *recA* strains of *E. coli*, indicating insertions in *recA*.

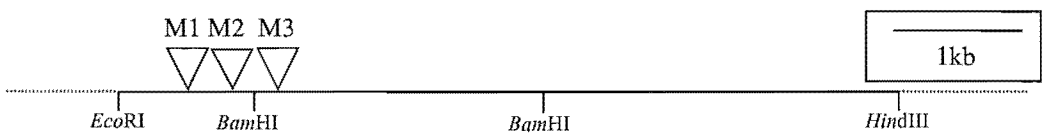


Figure 2.2. MiniTn*10* insertion points in the 6kb *Eco*RI/*Bam*HI *recA* region of pREC6g. The broken line indicates the vector pME6001.

pRECEB, pRECB, AND pRECBH.

In order to sequence the *P. aureofaciens recA*, three subclones of pREC6g were generated by ligation of *Eco*RI/*Bam*HI, *Bam*HI, or *Bam*HI/*Hin*dIII cut DNA with appropriately digested pBLUESCRIPT KS- (Stratagene). The subclones were designated pRECEB, pRECB, and pRECBH (Figure 2.3). With respect to the multicloning site in pBLUESCRIPT KS-, the cloned fragments are oriented as follows. The *Eco*RI/*Bam*HI fragment has the *Eco*RI site 5' relative to the *Bam*HI site. The *Bam*HI fragment in pRECB has the left most *Bam*HI site (as illustrated in Figure 2.3) at the 3' end, close to the T3 promoter-primer binding site. The *Bam*HI/*Hin*dIII fragment is oriented with the *Hin*dIII site 5' relative to the *Bam*HI site.

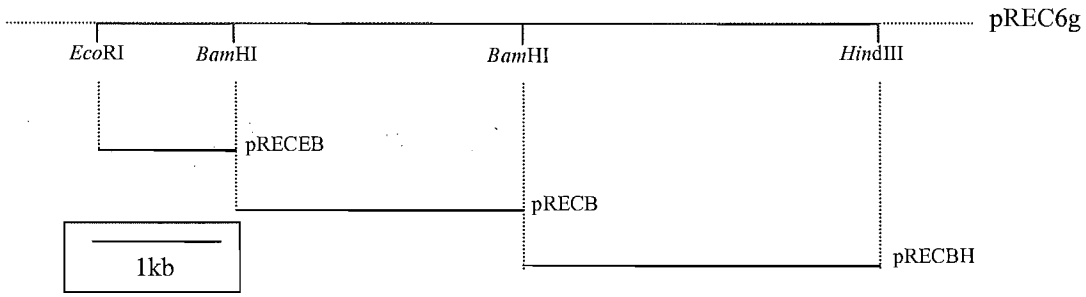


Figure 2.3. Subcloning of pREC6g to create the clones pRECEB, pRECB, and pRECBH. The horizontal broken line indicates the vector for pREC6g (pME6001).

pREC6L.

To construct a plasmid that would facilitate the creation of *recA* deletions in *P. aureofaciens*, a vector that is unstable in *P. aureofaciens* is desirable. For this reason, the 6kb *recA*-containing *Eco*RI/*Hin*dIII fragment was cloned from pREC6g into pLAFR3, creating the clone pREC6L which was used in further *recA* constructions.

pREC6LΔB.

This plasmid was constructed by digesting pREC6L at the two *Bam*HI sites, one of which is found within the *recA* coding region and the other is 2.4kb upstream of *recA*. The digested DNA was ligated to itself and used to transform *E. coli* DH5α. The transformants were screened for MMS^S, and then for loss of the 2.4kb *Bam*HI fragment.

pREC6LΔBg.

pREC6LΔBg was constructed by introducing the 2.4kb Gm^R *Bam*HI fragment from Tn5B61 into the unique *Bam*HI site in pREC6LΔB, thus introducing a selectable marker into the construct for use in allele exchange mutagenesis of *recA*.

2.7.3 Plasmids used in the study of *finR* (PA138).

pCM138OS and pCMsub.

pCM138OS is a *Sal*I subclone from pCM138E. Relative to pCM138, this clone has the other side of the Tn*5* from PA138, plus adjacent DNA (see Figure 2.4). pCMsub is a *Bam*HI/*Eco*RI subclone from pCM138E. It contains the Km^R gene from Tn*5*, and the complete *finR138* allele (see chapter 5).

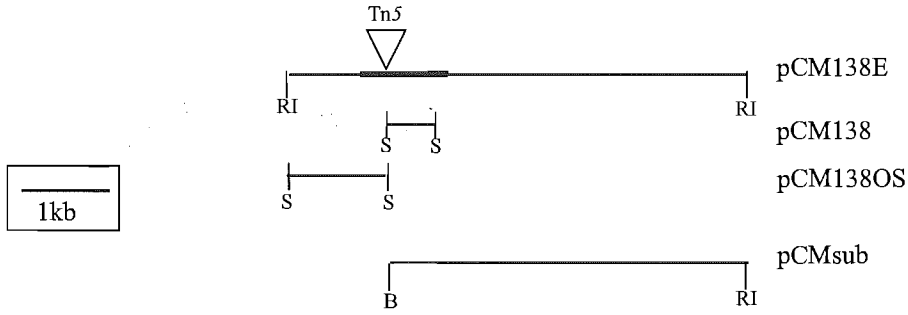


Figure 2.4. Construction of pCM138 subclones. The bold line indicates *finR*, and the triangle indicates the relative position of Tn*5* in *finR*. S=*Sal*I; B=*Bam*HI; RI=*Eco*RI.

pHRP309Cm.

The Cm^R Ω cassette from pHP45-ΩCm was cloned in the *Bam*HI site of pME6000, resulting in the plasmid pMEΩ. The Cm^R cassette was cloned from pMEΩ into pHRP309 using *Sal*I and *Eco*RI. pHRP309Cm has an omega cassette inserted adjacent to the start of the promoterless *lacZ*, to reduce read-through from plasmid sequences.

pFRTR833.

This plasmid was constructed by a three-step procedure. Firstly, an 833bp *SalI*/*Cla*I fragment from pCM138E was cloned in pBLUESCRIPT KS to create pFIN833. This digestion resulted in the subcloning of *finR*, from position 45 in the predicted *finR* coding sequence (*Sal*I side), and the *Cla*I recognition sequence begins at base pair 570 of the

predicted *finA* coding sequence. Thus, promoters for both *finR* and *finA* were likely to be within the 833bp. pFIN833 was digested with *Eco*RI and *Kpn*I, and cloned with identically digested pMEΩ. The resulting clone had the intact 833bp region adjacent to the ΩCm sequence, with the *finR* promoter oriented such that *finR* reads away from the ΩCm sequence. The final step in creating pFRTF833 was subcloning the entire 833bp plus ΩCm region into the *lacZ* fusion vector pHRP309, using *Kpn*I and *Xba*I. In pFRTF833 the *finR* promoter reads toward *lacZ*, resulting in a transcriptional fusion. Read through from the vector should be eliminated by the Ω cassette.

pFATF833.

pFATF833 was constructed in a similar way to pFRTF833. The 833bp region from pFIN833 was cloned into pHRP315 using *Eco*RI and *Kpn*I, resulting in plasmid pHRP833. The 833-bp insert in pHRP833 is adjacent to a ΩSm cassette, and the *finA* promoter reading away from the cassette. The entire 833bp plus the ΩSm was then cloned in pHRP309 in such a way that *finA* is oriented toward the *lacZ* gene. This was achieved using *Eco*RI and *Xba*I. The presence of the Ω cassette on the other side of the *finA* promoter (relative to *lacZ*) should prevent transcriptional or translational read-through from the vector.

pFATF1000.

pFATF1000 was constructed in a two-step process. Firstly, a *SalI*/*Eco*RI fragment from pMSA103 was cloned into equivalently digested pHRP833. pMSA103 is a *SalI* subclone from cosmid pPS192, and contains all of *finR* except the initial 39bp, which is contained in pHRP833. Thus, ligation of the *SalI*/*Eco*RI fragment into pHRP833 results in the reconstruction of *finR*, by joining the sequences at the *SalI* sites. Like pFATF833, the *finA* promoter reads away from the ΩSm cassette, which is found downstream of *finR*. This clone was called pHRP1000. pHRP1000 was digested to completion with *Eco*RI, and then partially digested with *Xba*I prior to ligation with *Eco*RI/*Xba*I digested pHRP309. The resulting *finA*::*lacZ* fusion construct was called pFATF1000.

pFATF2000.

To clone the *finR138* allele adjacent to the *finA* promoter in pHRP833, pCM138 was digested with *Sal*I and *Sma*I. pHRP833 was digested with *Xho*I, end-filled with Klenow, then digested with *Sal*I. The digests were mixed and ligated. The resulting clone, in which a *finR138* allele is reconstructed by ligation of the *Sal*I ends was named pHRP2000. This clone has *finA* promoter reading away from *finR138*, which has the ΩSm cassette downstream of it. pHRP2000 was digested to completion with *Eco*RI, then partially digested with *Xba*I. These fragments were ligated to *Eco*RI/*Xba*I digested pHRP309, resulting in the creation of pFATF2000. pFATF2000 has a *finA*::*lacZ* fusion with a *finR138* allele downstream. The entire *Eco*RI/*Xba*I insert DNA is approximately 6kb.

pDELKL.

pDELKL was generated in several steps, beginning with PCR amplification of the sequences flanking *finR*. As indicated in the Figure 2.5, one primer in each pair had a *Bam*HI recognition sequence included (see appendix 3).

pDEL1 and pDEL2 were constructed by PCR and cloning of the amplified DNA. After *Bam*HI digestion and ligation, PCR using primers 1 and 4 allowed the amplification of the *finR* deletion. Following construction of pDEL3, a Km^R gene (from pNK2859) was cloned into the *Bam*HI site, resulting in pDELKg. Finally, the *Eco*RI region from pDELKg was cloned in pLAFR3, creating pDELKL. The cloned *Eco*RI region has the complete deletion of *finR*, replaced with the Km^R gene.

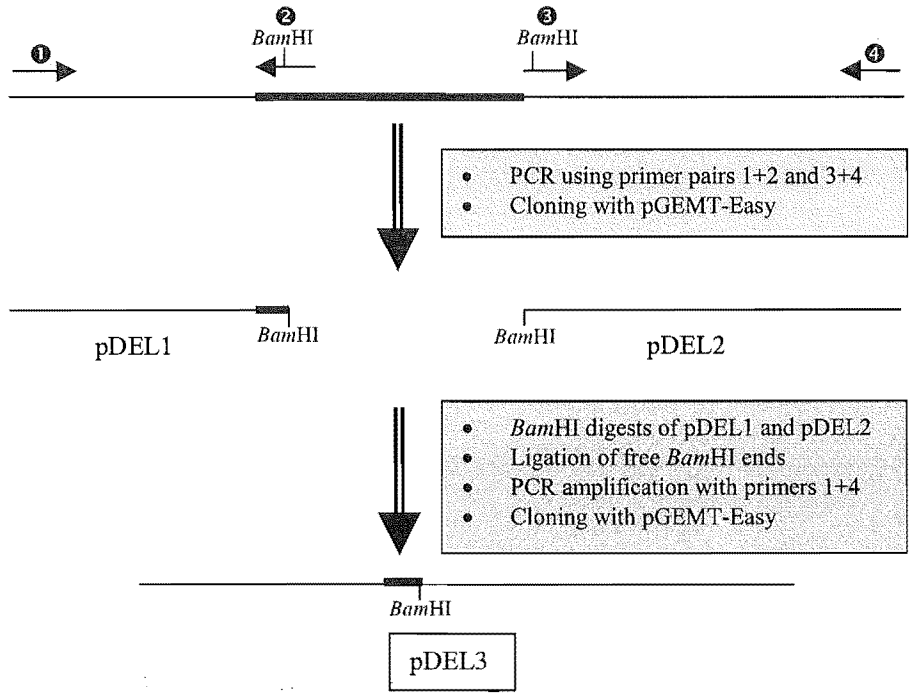


Figure 2.5. Construction of pDEL3. The thick line represents *finR*, while the thin horizontal lines represent flanking DNA. Horizontal arrows represent primers. 1, *finR*dFwd1; 2, *finR*dRev1a; 3, *finR*dFwd2; 4, *finR*dRev2

pRED2L AND pRED2LGM.

pRED2L was constructed by cloning a 2kb *Sal*I fragment that extends from base 45 in *finR* through *finA* to the next extragenic *Sal*I site. pRED2L contains the entire *finA* ORF. Initially the *Sal*I fragment was cloned in pUC18 (called pRED2). pRED2L was created by cloning the 2kb *Sal*I fragment from pRED2 into pLAFR3, using *Eco*RI and *Hin*dIII from the pUC18 multi-cloning site. pRED2Lgm was constructed by digesting pRED2L with *Cla*I, and using the Klenow fragment to fill in the overhanging ends. The Gm^R gene from the plasmid pJQ200 was isolated by *Hin*dIII digestion, and following end-filling using Klenow, the Gm^R encoding fragment was ligated with the linear pRED2L.

2.7.4 Plasmids constructed for the study of PA109 (*finT*).

pAF16M.

pAF16M was constructed by cloning the 16kb *Eco*RI fragment from cosmid pPS7138 in the vector pME6000. The 16kb region is that which is interrupted by transposon insertions in PA109 and PA1.

pAF9.

9kb *Bam*HI/*Eco*RI fragment from pAF16M cloned in pLAFR3.

pAF9::miniTn*10*.

Seven miniTn*10* insertion mutants of pAF9 were made. These were named pAF9.2, pAF9.38, pAF9.45, pAF9.55, pAF9.6, pAF9.66, and pAF9.68. The positions of these insertions within the 9kb are shown in Figure 2.6.

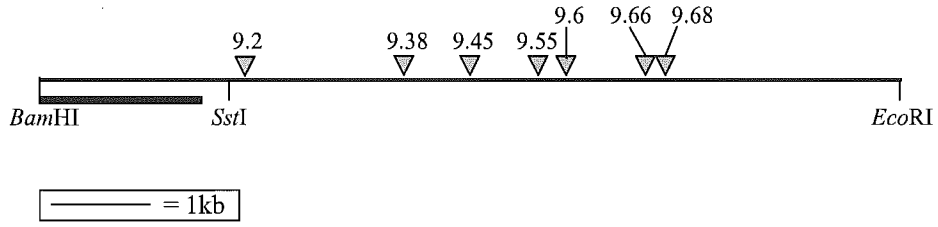


Figure 2.6. MiniTn*10* insertions in pAF9. Triangles represent miniTn*10*. Numbers above the triangles indicate the name of the particular plasmid with an insertion mutation. The bold line between *Bam*HI and *Sst*I indicates *finT*.

pAF9M, pAF8M, pAF7M, pAF6M, and pAF2M.

These five plasmids are subclones from pAF16M, cloned in the vector pME6000 for complementation experiments. Their construction is illustrated in Figure 2.7.

pAF2BK.

The clone pAF2BK was constructed by cloning the 2kb *Bam*HI/*Sst*I fragment from pAF2M with the vector pBLUESCRIPT KS-.

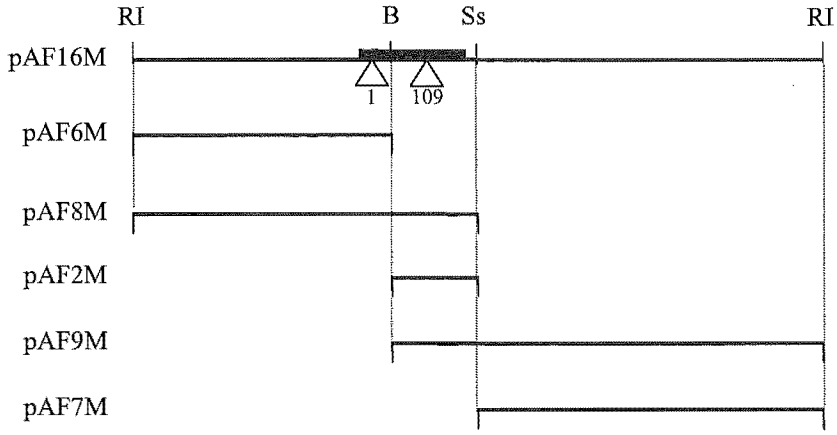


Figure 2.7. Construction of *finT* subclones. pAF9M, pAF8M, pAF7M, pAF6M, and pAF2M were created by subcloning from pAF16M. The number in each clone's name indicates the size of the cloned region in kb. RI=*Eco*RI; B=*Bam*HI; Ss=*Sst*I. For reference, the sites of transposon insertion in PA109 and PA1 are indicated with triangles. The position of *finT* is indicated by the bold horizontal line in pAF16M.

2.8 Protein labelling (^{35}S-cysteine and ^{35}S-methionine).

2.8.1 Maxicells.

GENERATION OF MAXICELLS.

In order to generate maxicells, the *E. coli* strain CSR603 was transformed by the appropriate plasmids. Cultures of CSR603 containing the plasmids were grown for 18 hours in LB, then subcultured 1:100 into K-medium (appendix 1) supplemented with Tc, and allowed to grow until an OD_{600} of 0.5 was reached. The bacteria were subcultured 1:50 in K-medium and allowed to grow to OD_{600} of 0.4.-0.5. Aliquots (2mL) were placed in Petri dish bases and exposed to UV light (250μJ/cm^2) using an Ultralum UVC515 ultraviolet multilinker. Immediately after irradiation, the bacteria were placed in foil-wrapped Universal bottles and incubated for one hour at 37°C. To assess the number of survivors, 50μL aliquots were spread onto LB plates, which were incubated for 24 hours. Generally this procedure yielded less than 20 cfu/mL after the UV treatment. Once the 50μL sample had been removed, fresh D-cycloserine solution was added (final concentration 100μg/mL) and incubation was continued for 16-18 hours.

LABELING PROTEINS EXPRESSED IN MAXICELLS.

Cells were collected by centrifugation and washed twice with sulphate-free Hershey salts before resuspending in 1.5mL of Hershey medium. The cultures were incubated for one hour (37°C) before the addition of ^{35}S-labelled methionine and cysteine (Promix, Amersham Pharmacia Biotech, 14mCi/mL) to a concentration of 20μCi/mL, and a further one hour incubation. Cells were harvested by centrifugation and suspended in 50-100μL dH_2O. An equal volume of 2x treatment buffer (appendix 1) was added, and the samples were then boiled for three minutes and used, or stored at -80°C until required.

2.8.2 Total soluble proteins.

Total soluble proteins expressed by PA147-2, PA109, and PA138 were labeled during growth under conditions known to support antifungal compound synthesis. The appropriate bacteria were grown for two days in 20mL of buffered PDB at 25°C before the addition of ^{35}S-labelled methionine and cysteine (20μCi/mL). Incubation was continued for a further hour before 1.5 mL of the bacteria were harvested by centrifugation, and washed in cold minimal salts to remove extraneous labeled amino acids. The bacteria were suspended in 100μL dH_2O and an equal volume of 2x treatment buffer. Samples were boiled for three minutes prior to protein separation by SDS-PAGE. Excess sample was held at -80°C for further use.

2.9 SDS-PAGE of labeled proteins.

2.9.1 SDS-PAGE.

Denaturing polyacrylamide gels were made according to recipes outlined in Biorad instruction manuals accompanying the protein electrophoresis systems. Recipes for buffers and gels are given in appendix 1.

SDS-PAGE was carried out using either a Biorad mini protean II or a Biorad protean II system. Gel casting apparatus was assembled as described by the manufacturer using 0.75mm spacers. The resolving gel was prepared and poured between the glass plates, then overlaid with water or water-saturated butanol to exclude oxygen and thus aid polymerisation. Once polymerisation was complete, the water or butanol was removed and

the exposed gel surface rinsed with dH_2O prior to pouring the stacking gel and inserting the appropriate well-forming comb. After one hour the gel and electrophoresis apparatus were assembled and the appropriate volume of Tris-glycine tank buffer was poured into the upper and lower reservoirs. The protein samples (with treatment buffer) were boiled for three minutes before loading between 10 and 50μL onto the gel. Proteins were subjected to electrophoresis at 150V using the Mini protean II system, or at 13mA for the stacking gel and 18mA through the resolving gel (per gel) when using the Protean II equipment. A wide range protein standard (Sigma) was used as a molecular weight marker.

After electrophoresis, gels were stained in Coomassie Brilliant Blue (R-250) for one hour, then destained in destain solution 1 (one to three hours) and destain solution 2 (12-16 hours) (see appendix 1).

2.9.2 Autoradiography.

After destaining, gels were rinsed in dH_2O, soaked in Amplify™ (Amersham) for 30 minutes, then rinsed in dH_2O again. Gels were dried onto Whatman number 1 chromatography paper for two to five hours using a Biorad model 443 slab dryer. Amersham hyperfilm-MP was exposed to the dried gel in an autoradiography cassette at -80°C for between one and four days. The film was then developed in AGFA G-150 developer for five minutes, followed by a one minute rinse in dH_2O. The film was fixed in AGFA G-334 fixer for two minutes, and rinsed in tap water.

2.10 DNA sequencing.

The nucleotide sequence of dsDNA was determined using an ABI Prism Model 377 Automated Sequencer utilizing Rhodamine terminator chemistry, or by using a LI-COR Model 4000L Automated DNA sequencer with infrared dye (IRD40) technology. All DNA sequencing utilised chain terminating dideoxynucleoside triphosphate (Sanger et al., 1977).

2.10.1 ABI Prism automated sequencing.

Automated sequencing using the ABI Prism 377 was carried out at the Waikato DNA sequencing facility (Department of Biological Sciences, The University of Waikato). This

system was used when primers other than the T7 and T3 promoter primers were required. DNA templates and primers were dissolved in sdH_2O and supplied at concentrations of 200ng/μL (12μL total) and 0.8pmol/μL (5μL total) respectively.

2.10.2 LI-COR automated sequencing.

When templates could be sequenced using T7 and/or T3 promoter primers, a LI-COR 4000L DNA sequencer was used.

CYCLE SEQUENCING REACTION.

DNA sequencing reactions were carried out using the Thermosequenase fluorescent primer cycle sequencing kit with 7-deaza-dGTP (Amersham Pharmacia biotech). Preparation of reaction mixes was carried out on ice, and tubes were covered with foil to minimize exposure to light. In 0.5μL microfuge tubes, template DNA (1pmol), IR-labelled primer (2pmol) and sdH_2O were mixed in a total volume of 17μL. Four 0.5μL tubes were labeled with A, T, G, and C, and 2.0μL of the appropriate thermosequenase reagent mix (contains dNTPs, the appropriate ddNTP, buffer and thermosequenase DNA polymerase) was added to each tube. Four microlitres of the template/primer mix were added to each tube and mixed, and the tubes were then overlaid with 50μL of PCR mineral oil. Reactions were cycled in a Hybaid Omnigene thermocycler (Appendix 2). Following the thermocycling reactions, 4μL of stop solution (formamide, EDTA, fuschin loading dye) were added. Reactions were held on ice or at -20°C until the electrophoresis step.

SEQUENCING GEL ELECTROPHORESIS.

The LI-COR gel casting apparatus (using 66cm glass plates) was assembled as described by the manufacturer. A 4% Long Ranger™ Gel Solution (FMC Bioproducts) was prepared and cast, after which a 32-well rectangular comb was added. After allowing two hours for polymerisation, the comb was removed and the wells washed with 1 x TBE. The apparatus was assembled and the gel was pre-run for one hour in 1x TBE running buffer, using version 2.31 Data Collection DEV7 software. Scanner control parameters are listed in appendix 2. Samples were denatured for three minutes at 95°C, wells were washed for a second time, and samples were loaded. Electrophoresis was carried out for 16 hours, and results were obtained using Version 2.30 Image Analysis program.

DNA SEQUENCE SIMILARITY SEARCHES.

DNA sequence data was compared to sequences in public databases using the Blastn and Blastx programs (Altschul et al., 1990; Altschul et al., 1997) accessible at the website of the National Centre for Biotechnology Information (http://www.ncbi.nlm.nih.gov/blast).

2.11 Southern transfer and hybridisation.

2.11.1 Transfer of DNA from agarose gels to nylon membranes.

DNA was transferred from 0.7-1.0% agarose gels and immobilised on Hybond N+ membranes using a Pharmacia Vacugene XL blotting system. The apparatus was assembled as described by the manufacturer, with the membrane pre-wetted in dH_2O. A vacuum pressure of 45mbar was applied and maintained throughout the transfer procedure.

TRANSFER FROM STANDARD AGAROSE GELS.

The DNA was partially depurinated by covering the gel with 0.25M HCl for ten minutes. The HCl was rinsed from the apparatus with dH_2O, and then the transfer and fixing was achieved in a single step by flooding the apparatus with 0.4M NaOH (alkaline transfer). After one hour the NaOH was removed, and the membrane was rinsed in 2xSSC (appendix 1). The membrane was either used immediately or wrapped in Glad Wrap™ and stored at 4°C until required. The efficiency of transfer was assessed qualitatively by staining the gel in ethidium bromide after the transfer was complete and examining it for remaining DNA.

TRANSFER FROM FIGE GELS.

As specified by the FIGE unit manufacturer (Biorad), FIGE gels were stained for 30 minutes in fresh ethidium bromide, irradiated with 60mJoules of UV (Ultralum UVC515 ultraviolet multilinker) to cleave large DNA fragments, then destained in 0.5x TBE. After photographing the gel, it was soaked in 0.4M NaOH for 15 minutes and then the DNA was transferred to Hybond N+ using 0.4M NaOH as the transfer solution. The transfer was carried out for four hours with a vacuum pressure of 45mbar. After transfer the membrane was washed in 2 x SSC and used as described below.

2.11.2 Probe labelling, hybridisation and signal detection using ECL™.

ECL™ was used routinely for all Southern detections except those involving membranes prepared from FIGE gels.

LABELING AND HYBRIDISATION.

Hybridisations were carried out using hybridisation tubes in a Hybaid mini-hybridisation oven. DNA transferred from standard agarose gels was hybridised with probes labeled with the non-radioactive ECL™ (enhanced chemiluminescence) kit (Amersham). Membranes were pre-hybridised in sufficient (approximately 0.1mL/cm^2 of membrane) ECL Gold hybridisation buffer for one hour at 42°C, during which time the probe was labelled. DNA to be labeled was generally a restriction fragment purified from an agarose gel. Approximately 100ng of DNA in 10µL sdH_2O in an Eppendorf tube was labeled by firstly denaturing by heating for five minutes in a boiling water bath and then cooled on ice for a further five minutes. After a brief centrifuge spin, 10µL of ECL labeling reagent was added and mixed. Immediately after, 10µL of glutaraldehyde was added and mixed. Contents of the tube were collected by brief centrifugation, and the mixture was incubated at 37°C for 15 minutes. Following this, the probe was added to the hybridisation tube containing the membrane and hybridisation buffer. Hybridisation took place for approximately 16 hours at 42°C.

WASHING AND SIGNAL DETECTION.

Following hybridisation, the hybridisation buffer was washed from the tube by replacing the buffer with 5xSSC and heating the oven to 55°C. Once the temperature had reached 55°C, the SSC was replaced by primary wash buffer (0.4% SDS, 0.2xSSC) which had been warmed to 55°C. The stringency washing was continued for a total 20 minutes, consisting of an initial ten minute wash followed by two five minute washes, using fresh, warmed primary wash buffer for each wash. The blot was removed from the tube and washed twice with 2xSSC, each wash for five minutes. Excess SSC was drained from the blot.

Signal was detected by exposing the labeled DNA side of the membrane to a mixture of equal volumes of detection reagents '1'and '2', as specified by the manufacturer. The detection reagents were drained from the membrane after one minute, at which time the

membrane was wrapped in gladwrap. Amersham Hyperfilm-MP was exposed to the membrane in an autoradiography cassette for two minutes, after which the film was developed. The result obtained allowed additional exposure times to be determined, depending on the strength of signal.

DEVELOPING EXPOSED HYPERFILM-MP.

In a darkroom, the film was agitated in AGFA G-150 developer for five minutes, and then rinsed in running water for one minute. After rinsing, the film was immersed in AGFA G-334 fixer for two minutes. The film was washed in water and allowed to drip-dry.

2.11.3 Probing FIGE blots using [α-^{32}P] dCTP-labeled probe DNA.

PREPARATION OF LABELLED PROBE.

Linear DNA extracted from an agarose gel was labeled with [α-^{32}P] dCTP using the Ready-To-Go™ DNA labeling kit (-dCTP) (Amersham Pharmacia biotech).

The contents of the reaction mix tube were reconstituted by adding 20μL of dH_2O, and the tube was then incubated on ice for 30 minutes. Linear DNA was denatured by heating for five minutes in a boiling water bath followed by immediate cooling on ice for five minutes. The denatured DNA was added to the reaction mix, followed by 1-3μL [α-^{32}P] dCTP (3000 Ci/mM) and dH_2O to make up the final volume to 50μL. The contents of the tube were mixed gently and then incubated at 37°C for 15 minutes.

HYBRIDISATION OF LABELED PROBE AND IMMOBILISED DNA.

The membrane was pre-wetted in 95% ethanol followed by brief immersion in dH_2O. The membrane was rolled and placed in a Hybaid tube with 10mL of pre-hybridisation buffer (preheated to 68°C) (appendix 1). The tube was incubated for at least one hour at 68°C in a Hybaid mini-hybridisation oven, and then the solution was replaced with preheated (68°C) hybridisation solution (appendix 1)containing labeled probe DNA. Hybridisation was carried out for 16 hours at 68°C.

After hybridisation, unbound probe was removed by three washes, all of which were carried out for 15 minutes at 68°C. The first wash solution was 100mL of 2 x SSC and 0.1% SDS, and the two subsequent washes were 100mL of 0.2 x SSC and 0.1% SDS. The washed membrane was dried and wrapped in Glad Wrap™. A Kodak Storage Phosphor screen was exposed to the membrane for four to 24 hours.

DETECTION OF SIGNAL FROM KODAK STORAGE PHOSPHOR SCREENS.

Exposed Kodak Storage Phosphor screens were scanned with the Molecular Dynamics Storm™ 840 system, (Scanner Control version 4.00, build 54), using the Phosphor screen scanner type at 740V. Data were viewed and manipulated using the Molecular Dynamics ImageQuaNT™ (version 4.2a, build 13) software package.

2.12 Uptake of inorganic phosphate ($[^{32}P]K_2HPO_4$).

Cells grown overnight in LB (18-24 hours) were harvested, washed in phosphate-free salts (PGTIPS), and then grown to exponential phase in either K10 with the desired concentration of Pi, or the appropriate phosphate defined medium. Exponentially growing bacteria were chilled on ice and then washed twice with PGTIPS and suspended in 600µL of PGTIPS supplemented with Tc (a translation inhibitor). Of this 600µL, 100µL was used to determine the viable cells per mL. $[^{32}P]K_2PO_4$ was added to 500µL aliquots of cells to a final concentration of 20µM (200mCi.mMol^{-1}), and 50µL samples were taken at one, two, and three minute intervals. Each 50µL sample was immediately mixed with 500µL of PMM salts (excess unlabelled Pi) in order to effectively halt uptake of labeled Pi by providing a high concentration of unlabelled Pi to dilute labelled Pi to negligible levels. Samples were then applied to Millipore filters (0.22µm pore size) by filtration, and the immobilized cells were washed twice by filtering through minimal salts (5mL per wash). Filter membranes were removed and placed in scintillation vials containing 5mL of scintillant fluid (Packard Ultima gold). Pi-uptake was measured using a Wallac 1410 liquid scintillation counter (Pharmacia) to determine beta counts per minute.

By constructing a standard curve using beta counts produced by known amounts of $[^{32}P]K_2PO_4$ it was possible to convert the beta counts per minute from the samples into

amount of Pi uptaken. Standard curves were constructed at the same time as the assays for which they would be used were carried out. Viable, culturable cell counts for each test culture were used to calculate the amount of ^{32}Pi uptake per 1 x 10^8 bacterial cells.

2.13 Alkaline phosphatase assays.

2.13.1 Qualitative assay.

Qualitative assays for alkaline phosphatase were carried out by adding BCIP to the appropriate agar plate. BCIP is a chromogenic substrate for alkaline phosphatase, and when cleaved by a bacterium producing APase, produces distinctive blue colonies.

2.13.2 Quantitative assay.

Quantitative assays were carried out using broth cultures of bacteria grown in the appropriate liquid medium. Cultures were grown to either exponential phase or overnight (16 hours). After the OD_{600} was determined, 500μL aliquots of cells were treated with 20μL of chloroform and 10μL of 0.1% SDS and incubated at ambient temperature for ten minutes to partially disrupt the cell membrane. After disruption of the membrane, the pH was adjusted by the addition of 200μL of 1M Tris-HCl (pH8). p-Nitrophenol phosphate, a chromogenic substrate for alkaline phosphatase, was added at (20μL of a 50μM stock). The reaction was allowed to proceed at ambient temperature, and was stopped by adding 100μL of 200mg/mL glycerol-3-phosphate once sufficient yellow colouration had developed. After separation from the cell debris by centrifugation (ten minutes, 17000 x g, ambient temperature) the yellow reaction product was measured at OD_{410}. Alkaline phosphatase activity is expressed as nM p-nitrophenol produced per minute per OD_{600}. The amount of p-nitrophenol produced was calculated with the relationship that 0.05μM.mL^{-1} gives an OD_{410} reading of 0.89.

2.14 β-galactosidase activity assay.

Assays for β-galactosidase activity were carried out essentially as described (Miller, 1972). Cultures were grown in the appropriate medium for the desired length of time, following which 1mL of cells were collected by centrifugation, and washed with cold minimal salts.

The cells were resuspended in 1mL of minimal salts. An OD_{600} reading was taken from 900μL of cell suspension and the remaining 100μL of the suspension was added to 900μL of Z-buffer. Two drops of chloroform and one drop of 0.1% SDS were added to disrupt cell membranes. Following ten seconds of vortex mixing, the lysed culture was equilibrated at 30°C for ten minutes, before the addition of 200μL of ONPG (4mg/mL, dissolved in Z-buffer). The reaction was left until a yellow colour developed, after which it was stopped by adding 500μL of 1M Na_2CO_3. The time in minutes was recorded. The reaction sample was centrifuged to remove cellular debris (ten minutes, 17000 x g, ambient temperature), and the OD_{420} of the solution was measured. β-galactosidase activity was calculated in Miller units using Equation 2.1.

$$\text{Units} = 1000 \times \frac{OD_{420}}{t \times v \times OD_{600}}$$

Equation 2.1. Formula for calculation of β-galactosidase activity in Miller units (Miller, 1972). t = time of assay (minutes); v = volume of cell suspension used in the assay (mL); OD_{600} is the cell density before the assay; OD_{420} is the reading of the yellow colour from the assay.

2.15 Insertional mutagenesis with miniTn*10*.

Insertional mutagenesis using miniTn*10* elements was carried out essentially as described (Kleckner et al., 1991). The miniTn*10* elements used in this study were carried on phage λ.

GROWING PHAGE λ LYSATES.

A high titre phage stock (10^{10} cfu/mL or greater) was prepared as follows. An LB plate was overlaid with H-Top agar containing 100μL of a saturated culture of *E. coli* PB2480 (a permissive host) grown in LBMM. Phage stock was 10-fold serially diluted and 10μL aliquots of the dilutions were applied to the plate. After overnight incubation at 37°C, a single well-isolated phage plaque was picked and transferred to 10mL of LBMM broth containing 100μL of a fresh saturated PB2480 culture (grown in LBMM). This culture was allowed to grow for five to six hours with vigorous shaking (350rpm) until it had visibly cleared. To maximise cell lysis, 100μL of chloroform was added, and the culture was shaken before being allowed to settle for ten minutes at ambient temperature. Cellular

debris was removed by centrifugation, and the supernatant was transferred to a sterile Universal bottle for storage at 4°C.

GENERATING INSERTIONS INTO PLASMIDS.

The plasmids to be mutated were used to transform *E. coli* MC4100, a *supO* strain that is not permissive for replication of the phage λ vehicle. The transformants were grown to saturation in LBMM, and concentrated to approximately 10^{10} cells per mL in fresh LB. Phage were added to the bacteria at various multiplicities of infection (range from 0.1 to 1) and allowed to adsorb for 15 minutes at ambient temperature followed by 15 minutes at 37°C. Cells were washed in LB containing sodium citrate (50mM) and grown in LB plus citrate for one hour, after which they were plated on LB supplemented with sodium pyrophosphate (1.25mM) and appropriate selection for the presence of the plasmid and transposon. The colonies were washed off the plate and plasmid DNA was prepared from them. The plasmid DNA was used to transform MC4100. Transformants were selected on LB supplemented with antibiotics to select plasmids bearing a transposon insert. Transformants were screened for the desired phenotype, or plasmids were extracted and the site of insertion was determined by restriction mapping.

2.16 Field trial methods.

2.16.1 Treatment of crowns.

PA147-2 was cultured at 30°C in LB media to a viable count of 1 x 10^8 CFU ml^{-1} (250 rpm, 24 h). The culture was maintained on ice during transportation to the field. Asparagus crowns were inoculated by submergence in a suspension of PA147-2 for 25 minutes with gentle agitation. Untreated controls were dipped in sterile distilled water (dH_2O), and LB treatment was carried out by dipping crowns in the medium. Crowns were planted within 30 minutes of treatment.

2.16.2 Bacterial population study.

Aerobic, Rf^R bacteria were isolated at two-week intervals from the rhizosphere and soil surrounding the rhizosphere (non-rhizosphere) from both PA147-2 inoculated and control

crowns within guard rows. Random soil samples of 1g each were collected from each sample area and placed in 100 mL of sterile dH_2O containing 10g of sterile gravel (7mm coarse chip). Following agitation (250 rpm for 20 minutes), serial 10-fold dilutions were spread-plated on LB agar supplemented with Rf and Cx and incubated (30°C for 24 h). Culturable rifampicin resistant (Rf^R) CFU g^{-1} were determined for rhizosphere and non-rhizosphere samples and the mean determined for the four samples.

2.16.3 Rf^R isolate analysis.

The following procedures used a -80°C stock of the original PA147-2 inoculation culture as a comparative control in all experiments. Colony morphology of Rf^R isolates was compared using a stereo microscope and cellular morphology was observed by Gram staining. Bioassays against *P. megasperma* var. *sojae* were carried out as described (Carruthers et al., 1994). Fluorescent siderophore production was determined by incubation (30°C for 72 h) of isolates on Pseudomonas Minimal Media (PMM) followed by viewing under UV illumination (260 nm). Antibiotic resistance of isolates was determined by replica-plating onto LB agar supplemented with either Rf, Ap, Km, Gm, Nal, Sm or Tc and following incubation for 24-72 h at 30°C, bacterial growth was assessed. Analytical profile index (API) 20 NE strip analysis was performed according to the manufacturer's protocol (Bio-merieux).

2.16.4 Harvest statistical analysis.

After harvest, individual plants were visually graded on percentage of diseased foliage using a scale of 1 – 5 (1 = 0%, 2 = 1-20 %, 3 = 21–40%, 4 = 41-80%, 5 = 81–100%) and analysed by fitting an ordinal logistic model. Fern dry weights for each treatment were analysed for significant differences using a one-sided t-test.

2.17 Construction of *lacZY* marked PA147-2.

The *lacZY* genes from *E. coli* were introduced into PA147-2 as described (Barry, 1988). pMON7197 containing a modified Tn*7* element bearing *lacZY* was mobilised into PA147-2 by conjugation. *P. aureofaciens* strains bearing a transposon insertion were selected by the

ability to grow on lactose minimal medium (M9 supplemented with 0.4% lactose as the sole carbon source).

2.18 Competition experiments.

Competition experiments were carried out in 20mL of PMM at 25°C. Competition cultures were inoculated with 1:200 dilutions of the appropriate bacteria grown for 20 hours in LB. The 20 hour old inoculum was started from stock held at -80°C. The competition cultures were sampled every 24 hours, and these samples were 10-fold serially diluted. Plating of serial diluted cultures on PMM plus Xgal, PMM plus Km, and M9 lactose medium allowed enumeration of Lac^+ and Km^R bacteria in addition to counts of blue and white colonies on PMM, supplemented with Xgal.

2.19 Amplification of DNA by the polymerase chain reaction (PCR).

Target regions of the *P. aureofaciens* PA147-2 genome were amplified using AmpliTaq Gold (Perkin-Elmer). PCR was carried out in 0.5mL, thin-walled tubes, in total reaction volumes of 25μL. The composition of the reaction included 1μL of template (either bacterial total DNA, or a suspension of bacterial cells), 2.5μL 10x PCR buffer, 0.5μL dNTPs (from a 10mM stock), 10pmol of each primer, 0.5μL of AmpliTaq gold, and an empirically determined concentration of $MgCl_2$. Reactions were made up to the total volume with sdH_2O, and overlaid with mineral oil. Thermal cycling was carried out in a Hybaid omnigene, using the programs listed in appendix 2. Following completion of the amplification, PCR products were examined using agarose gel electrophoresis, and purified as required for further use.

2.20 Reverse transcription PCR (RT-PCR).

2.20.1 RNA preparation.

RNA was prepared from PA147-2 using the SV total RNA isolation system (Promega), following manufacturer's recommendations. The RNA extracts were subjected to an additional DNase treatment using RNase-free DNase (Life technologies) followed by

phenol/chloroform and chloroform extractions. Finally, RNA was precipitated using three volumes of ethanol and 1/20 volumes of 5M NaCl, and was washed with 70% ethanol.

2.20.2 RT-PCR.

RT-PCR was carried out using Ready-to-go RT-PCR beads (Amersham Pharmacia biotech). The manufacturer's instructions for one-step RT-PCR were followed. A control reaction was carried out in which reverse transcriptase was inactivated by heating at 95°C for 10 minutes prior to the reverse-transcription step. No DNase-treatment control was used since the RNA had been DNase treated twice, and such a control was not suggested to be necessary by the manufacturer . Reactions consisted of 10pmol of each primer, 500-1000ng of RNA, and the contents of the Ready-to-go bead (M-MuLV reverse transcriptase, Taq polymerase, reaction buffer, dNTPs, RNase inhibitor). Reaction volumes were made up to 50μL with diethyl pyrocarbonate (DEPC)-treated sdH_2O, and cycled in a Hybaid omnigene thermal cycler (see appendix 2 for program details). Amplified DNA was visualised by agarose gel electrophoresis.

2.21 N-terminal protein sequencing.

2.21.1 Transfer of proteins to PVDF membrane.

SDS-PAGE was carried out as described above. Once complete, the proteins were transferred to PVDF membrane (Biorad) using a Biorad mini trans-blot® electrophoretic transfer cell, according to the manufacturer's instructions. The gel was allowed to equilibrate in the transfer buffer (25mM Tris, 192mM glycine, 20% v/v methanol) for one hour. To wet the PVDF membrane, it was submerged in methanol for five seconds, and then transferred to a container of transfer buffer. Membrane saturation was apparent when the membrane stopped floating on the buffer surface. Two pieces of Whatman number 3 filter paper cut to the size of the gel were saturated with transfer buffer, as were two fibre pads. A "transblot sandwich" was prepared by placing items in the following order into the gel holder cassette. First, a fibre pad was laid onto the holder. One piece of filter paper was layered onto the pad, and then the gel was carefully layered onto the paper. The pre-wet PVDF was placed onto the gel, ensuring that there were no air bubbles trapped between the gel and membrane. Filter paper and finally the second fibre pad were layered on top of

the membrane. The cassette could then be closed, placed in the transfer tank, and covered with cold (4°C) buffer. The transfer was carried out at 30V for 16 hours, in a 4°C refrigerator. The buffer was continually circulated using a magnetic stirrer.

After the transfer was complete, the PVDF membrane was removed from the apparatus, rinsed in dH_2O, and saturated in methanol for five seconds, prior to staining. Protein on the membrane was detected by staining with Coomassie brilliant blue R250 (see appendix 1 for recipe). After saturating the membrane in methanol, it was transferred to the Coomassie blue stain, and allowed to stain for one minute with constant orbital shaking. Immediately after the staining, the membrane was transferred to 50% methanol to destain. Several changes of destain were required to properly visualise the proteins of interest.

2.21.2 N-terminal sequence determination.

The protein of interest on the stained membrane was marked on the membrane with pencil. The membrane was carefully packaged and sent to The University of Auckland Protein Analysis Facility for N-terminal sequence determination using an Applied Biosystems Procise sequencer. N-terminal sequence obtained from this procedure was used to search the Swissprot database for similar proteins, using the ExPASy www server (http://expasy.proteome.org.au/tools/scnpsit2.html).

2.22 Abiotic biofilm assay.

Abiotic biofilm assays were carried out essentially as described (O'Toole & Kolter, 1998b). Briefly, the wells of PVC microtitre plates were filled with 98μL of the appropriate medium, and then inoculated with 2μL of bacteria from an overnight culture. The plate was covered and incubated at 30°C for 16 hours. Following incubation, 25μL of 1% crystal violet solution was added to each well and allowed to stain for 20 minutes, after which the wells were vigorously rinsed with tap water. Biofilm formation was indicated by the presence of a ring of stained bacteria adhered to the well at the oxygen/medium interface.

Chapter 3

Construction of *recA* strains of *Pseudomonas aureofaciens* PA147-2 and its mutants

3.1 Introduction.

3.1.1 The utility of complementation.

Much of the knowledge regarding the molecular basis of phenotypes is derived from the study of mutants. When examining such mutants it is useful to confirm that the mutation under investigation is indeed the cause of the altered phenotype, and that the phenotype is not a consequence of some other unknown mutation. This can be accomplished in a number of ways. Reconstruction experiments can be carried out by (i) isolating the mutated region of chromosome and using it to recreate the mutation by homologous recombination, or (ii) transferring the mutation to the parental strain by generalised transduction. If the recreated mutant shows the original mutant phenotype one can proceed confidently with analysing the mutant. Another approach is to isolate a complementary DNA sequence obtained from a genomic library and use it to replace the mutated region by homologous recombination. If the mutation was the genuine cause of the observed phenotype, the allele replacement should restore the wildtype properties. As databases of DNA sequence become annotated with functional information some mutants may become amenable to a purely bioinformatic approach. The DNA sequence surrounding the mutation may be similar to known genes, and may allow a correlation with the observed phenotype. Examples where this approach might be sufficient include analysis of an auxotroph, or investigation of a mutant deficient in a well-studied property such as flagella synthesis. The obvious limitation with the bioinformatic approach is the assumption that sequence similarity gives functional identity. A fourth method used to confirm the cause of a mutant phenotype is *trans* complementation. This method assesses the ability of a wildtype DNA sequence complementary to the mutated region to restore the wildtype phenotype without replacing the mutated region. *Trans*-complementation has some advantages over the other approaches outlined here. Because *trans* complementation does not replace the mutated region, it requires the complementing sequence to be genetically sufficient to restore the wildtype phenotype to the mutant. Thus, in addition to confirming

the nature of the observed phenotype, *trans* complementation provides additional information regarding the presence of intact genes or operons within the complementing sequence. This is in contrast to allele exchange in which the mutated sequence is replaced by the wildtype allele. While giving the same confirmation as *trans* complementation with a wildtype sequence, allele exchange can be achieved with a DNA region that is not itself sufficient to complement the mutation. All that is required is that the exchanged sequence has DNA from both sides of the mutation, allowing recombination to replace the mutated region, whereas an intact gene or operon is necessary for *trans* complementation to be observed. Probably the most powerful use for *trans* complementation is in combination with the other approaches outlined. For example, a comparison between *trans* complementation and allele exchange has the power to give insight into phenomena such as *cis/trans* requirements of the mutated genes, and polar effects arising from the mutation.

3.1.2 Complementation in *Pseudomonas aureofaciens* PA147-2.

In a previous study (Carruthers, 1994), it was reported that *trans* complementation was not possible in PA147-2 mutants due to a high rate of homologous recombination and vector instability. This was a particular problem when cosmids were used since they have large genomic regions for recombination to occur and the vector pLAFR3 is unstable in PA147-2 in the absence of antibiotic resistance selection. To circumvent the problem, allele exchange was used to replace the mutation with the wildtype sequence (Carruthers et al., 1994), a technique which has also been employed in the present study (chapter 5). Given the potential benefits of using *trans* complementation, the development of a system that would permit *trans* complementation was undertaken. Although some cloning vectors that are stable in *Pseudomonas* spp. are available (Stephen Heeb, Pers Comm; Heeb et al., 2000), their use for *trans* complementation would be limited because vector stability was only part of the problem. Additionally, the use of new vectors would not alleviate the problem with respect to cosmid complementation since the PA147-2 genomic library is constructed in pLAFR3. A new library could be constructed in a stable vector, but since this would only partially solve the problem, it was decided that a better approach would be to develop a system that would allow complementation in *trans*. To this end, it was decided to create a *recA* defective strain of PA147-2.

3.1.3 *recA.*

The recombination deficient phenotype involving *recA* in *E. coli* was first discovered during Hfr mating experiments, when it was noted that a recipient strain inherited nutritional markers at a lower than expected frequency (Clark & Margulies, 1965). The defect was shown to be in homologous recombination between incoming DNA and the recipient genome. RecA is now known to be a multifunctional protein in *E. coli*, with well-characterised roles in homologous recombination (Kowalczykowski et al., 1994), recombinational DNA repair, and induction of the SOS regulon (Miller & Kokjohn, 1990). In the first two of these processes, RecA promotes homologous pairing and strand exchange, while in the SOS response RecA acts as a co-protease in the cleavage of the LexA repressor of the SOS regulon. RecA also appears to function in homologous recombination and DNA repair in *P. fluorescens* (De Mot et al., 1993) *P. putida* (Luo et al., 1993) and *P. aeruginosa* (Sano & Kageyama, 1987). In addition to the functions above, *recA* has been shown to be involved in the secretion of an extracellular nuclease by *Serratia marcescens* (Ball et al., 1990), and in the regulation of colicin E1 expression in *E. coli* (Salles et al., 1987). Construction of *recA* mutant strains of *P. syringae* (Willis et al., 1988) and *P. putida* (Luo et al., 1993) resulted in strains that were useful in *trans* complementation studies.

The interest in the *recA* system of *P. aureofaciens* PA147-2 was two-fold. Firstly, since the loss of cosmids has often been accompanied by allele exchange between the cosmid and the genome, it was of interest to examine any possible role of RecA in this phenotype. Secondly, construction of *recA* mutants might facilitate further genetic analysis of PA147-2 and its derivatives by stable in *trans* complementation experiments, since this has been a successful approach in related pseudomonads.

3.1.4 Objectives.

The main objective of the work reported in this chapter was to create *recA*-deficient strains of PA147-2 and to assess the usefulness of these strains as a *trans* complementation system. To achieve this, several aims were defined, and are as follows.

1. Recover a *recA*+ cosmid from the PA147-2 library.

2. Use miniTn*10* mutagenesis to disrupt *recA* in a cosmid and introduce the mutated *recA* into the PA147-2 genome by homologous recombination.
3. Assess the effect of disrupting *recA* on cosmid stability and recombination frequency.
4. Develop a system that would allow *recA* to be mutated in the Tn*5* mutants of PA147-2.

3.2 Scope of this chapter.

This chapter contains the development of a *trans* complementation system in PA147-2. The introduction has outlined the background and rationale for this work, and provides the objectives and aims of the work. Following this section, the chapter describes (i) the isolation and mutagenesis of a *recA* cosmid from the genomic library of PA147-2; (ii) the demonstration that the PA147-2 *recA* can restore *recA*-dependent functions to *recA* mutants of *E. coli*; (iii) the construction of two *recA* mutants of PA147-2 by miniTn*10* mutagenesis of the *recA* cosmid and allele exchange in PA147-2. These strains (PA147-2*recA*1 and PA147-2*recA*4) were used to test the stability of cosmids in *recA* mutants. The research up to this point was published in the Canadian Journal of Microbiology (Silby & Mahanty, 2000). Following the published work, the DNA sequence of the PA147-2 *recA* is shown, and the development of a system to create partial *recA* deletions in the Tn*5*-generated antifungal mutants of PA147-2 is described. All clones and mutagenised plasmids created in this work are described in section 2.7.2.

3.3 Results and Discussion.

3.3.1 Isolation, subcloning and mutagenesis of PA147-2 *recA*.

Isolation of a recA cosmid.

The ability to provide *recA*-defective *E. coli* strains with resistance to the DNA-damaging agent MMS has previously been associated with complementation by heterologous *recA* clones (Better & Helinski, 1983). The genomic library of PA147-2 (stored in *E. coli* DH5α(*recA*$^-$)) was plated on LB plates supplemented with tetracycline and MMS. Six colonies grew after overnight incubation, and these were all shown to contain identical cosmids (data not shown). One of the cosmids (pREC1) was used to transform a *recA* mutant of *E. coli* ZK4 to MMS resistance, thus confirming the ability to confer MMS resistance and lending support to the possibility that pREC1 contained *P. aureofaciens recA*.

A 6kb *Eco*RI/*Hin*dIII fragment from pREC1 was subcloned into pME6001 to produce pREC6g, and both pREC1 and pREC6g were mutagenised with miniTn*10* (Kleckner et al., 1991) resulting in the clones pPRAM1 (pLAFR3-based), pPRAM4 (pLAFR3-based), pREC6M1 (pME6001-based), pREC6M2 (pME6001-based), and pREC6M3 (pME6001-based).

3.3.2 The MMS^R clone from PA147-2 complements *recA* mutations in *E. coli*.

Having isolated and subcloned a DNA region from PA147-2 that can confer MMS resistance upon *recA* strains of *E. coli*, it was necessary to show that the clone did indeed contain *recA*. In order to confirm this, pREC6g was tested for its ability to complement a number of RecA-dependent functions in *E. coli*.

Induction of the SOS response in *E. coli*.

RecA plays a critical role in the SOS response in *E. coli*, acting as a catalyst in cleavage of the LexA protein and thus derepresses the SOS regulon. Expression of *sulA* is induced in *E. coli* by the SOS response and SulA arrests cell division through interaction with FtsZ (Bi & Lutkenhaus, 1993). We tested the ability of the PA147-2 *recA* to induce an SOS response

by measuring expression of the *sulA* gene in *E. coli* strains DE1491(*recA*) and DE880(*recA*+). DE1491 and DE880 have a Mud*lac* insertion into *sulA*, creating an in-frame *sulA*::*lacZ* fusion. The expression of *sulA* can therefore be inferred by measuring the β-galactosidase level according to the method of Miller (1972). When pREC6g(*recA*+) was present in DE1491, an increase in *sulA* expression was observed, relative to DE1491 harbouring either pME6001 or pREC6M3(*recA*). The expression of *sulA* was further increased when the SOS response was induced by exposure to the DNA-damaging agent mitomycin C for 60 minutes prior to the β-galactosidase assay. In contrast to these observations, the presence of either pME6001 or pREC6M3 in DE1491 had no impact on *sulA* expression with or without addition of mitomycin C (Figure 3.1). When *E. coli* DE880, an otherwise isogenic *recA*+ strain of DE1491, was assayed under the above conditions, the level of β-galactosidase produced after mitomycin C exposure was comparable to that from DE1491(pREC6g) (Figure 3.1).

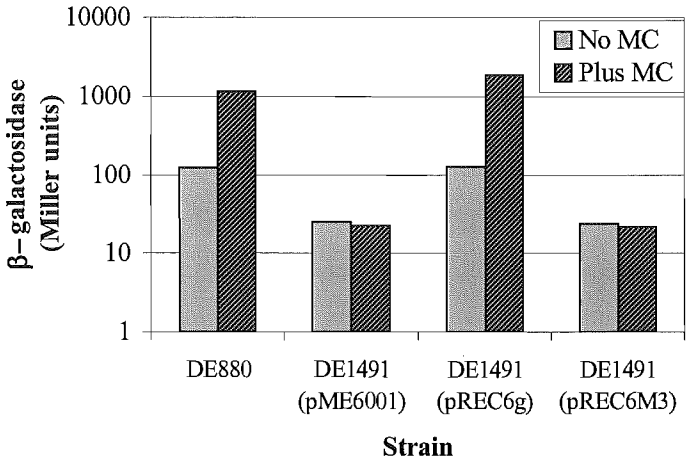


Figure 3.1. Induction of the SOS response in *E. coli* DE880(*recA*+) and DE1491(*recA*) and its derivatives. The β-galactosidase level from DE1491 harbouring the plasmids pREC6g(*recA*$^+$), pME6001, or pREC6M3 (*recA*) was measured, with and without mitomycin C (MC) exposure. The *recA*+ strain DE880 was included as a control. Both *E. coli* DE1491 and DE880 have a *sulA*::*lacZ* fusion, so β-galactosidase levels reflect the level of SulA, which is induced in the SOS response. Data are the average of four experiments. Standard error was less than 10%.

Resistance to DNA damage induced by UV and MMS.

E. coli strains DH5α and MC4100 are sensitive to exposure to UV and MMS, due to DNA damage. Resistance to UV and MMS was assessed qualitatively by the ability of bacterial strains to survive treatment with these DNA damaging agents. When pREC6g was present

in *trans*, both strains showed an increased resistance to UV and MMS, as measured by their ability to grow after UV exposure or in the presence of MMS. However, this increase in resistance was not detected when pREC6g(*recA*+) was replaced with either pME6001 or pREC6M3(*recA*). Neither strain could form colonies after UV exposure or on MMS supplemented plates, indicating a role for the PA147-2 *recA* gene in the provision of UV and MMS resistance, presumably by the restoration of RecA-dependent DNA-damage repair systems. *P. aureofaciens* PA147-2 *recA* mutants (described below) showed increased sensitivity to both UV and MMS, and resistance was restored by complementation with pREC6g, but not with pME6001 or pREC6M3 (data not shown).

Promotion of homologous recombination by pREC6g(recA+).

The ability of the MMS resistance-encoding clone pREC6g to promote homologous recombination was tested by P1 transduction of functional amino acid biosynthetic *his* and *ilv* alleles to a *his ilv E. coli* mutant (*E. coli* MT2). $P1_{vir}$ stock was grown on *E. coli* W3110, and used to transduce the auxotrophic *E. coli* strains (Miller, 1992). Inheritance of either nutritional marker was used to indicate recombination between the transduced DNA and the genome of the recipient. Strains (*E. coli* MT2) containing pREC6g showed inheritance of transduced markers whereas transductants of the control strains harbouring either pME6001 or pREC6M3 were not detected (Table 3.1). No spontaneous revertants of MT2 to either His+ or Ilv+ were detected (data not shown).

The primary motivation for this study was to investigate the possibility that disruption of *recA* in PA147-2 might alleviate the problem of recombination during *trans* complementation experiments. Since genetic systems are not well developed in *P. aureofaciens*, an *E. coli* system was used to test homologous recombination frequency. The data show that the wildtype *recA* clone of PA147-2 (pREC6g) can promote homologous recombination in *E. coli*, and an insertional mutant (pREC6M3) was unable to promote this RecA-dependent function (Table 3.1).

Table 3.1. Promotion of homologous recombination.

Strain	Inheritance of *his*[a]	Inheritance of *ilv*[a]
MT2 (pREC6g)	3.7×10^{-6}	9.5×10^{-5}
MT2 (pREC6M3)	$< 2.6 \times 10^{-7}$	$< 2.6 \times 10^{-7}$
MT2 (pME6001)	$< 1.8 \times 10^{-7}$	$< 1.8 \times 10^{-7}$

Notes: Homologous recombination is inferred from the frequency of inheritance.
[a] Inheritance is expressed as the number of cells inheriting the particular allele out of the total population of cells exposed to the transducing phage.

3.3.3 Introduction of *recA*::miniTn*10* mutations into the chromosome of PA147-2.

The two independently mutated plasmids designated pPRAM1 and pPRAM4 were used to introduce *recA*::miniTn*10* into the chromosome of PA147-2. Both pPRAM1 and pPRAM4 were transferred to PA147-2 by conjugation, and the mutated *recA* alleles were introduced to the genome of PA147-2 by homologous recombination. To allow recombination to take place, the transconjugants were grown without selection in LB for ten hours prior to plating on LB supplemented with Rf and Km. Km^R colonies were either recombinants (with or without the plasmid), or strains that had not undergone recombination and had not lost the plasmid. This procedure resulted in the strains PA147-2*recA1* and PA147-2*recA4*, both of which exhibit a loss of resistance to UV irradiation and MMS exposure. Recombination (rather than plasmid retention) was indicated by the loss of the vector marker (Tc^R) and was subsequently confirmed by Southern analysis (Figure 3.2), indicating the successful construction of *recA* mutants of PA147-2. The two mutated cosmids pPRAM1 and pPRAM4 were confirmed as having different insertion points by subcloning and DNA sequencing of the subclones pPRAM1.1 and pPRAM4.1 using a primer designed to anneal to the IS*10* of the miniTn*10* element (appendix 3). These data are shown diagrammatically in Figure 3.3.

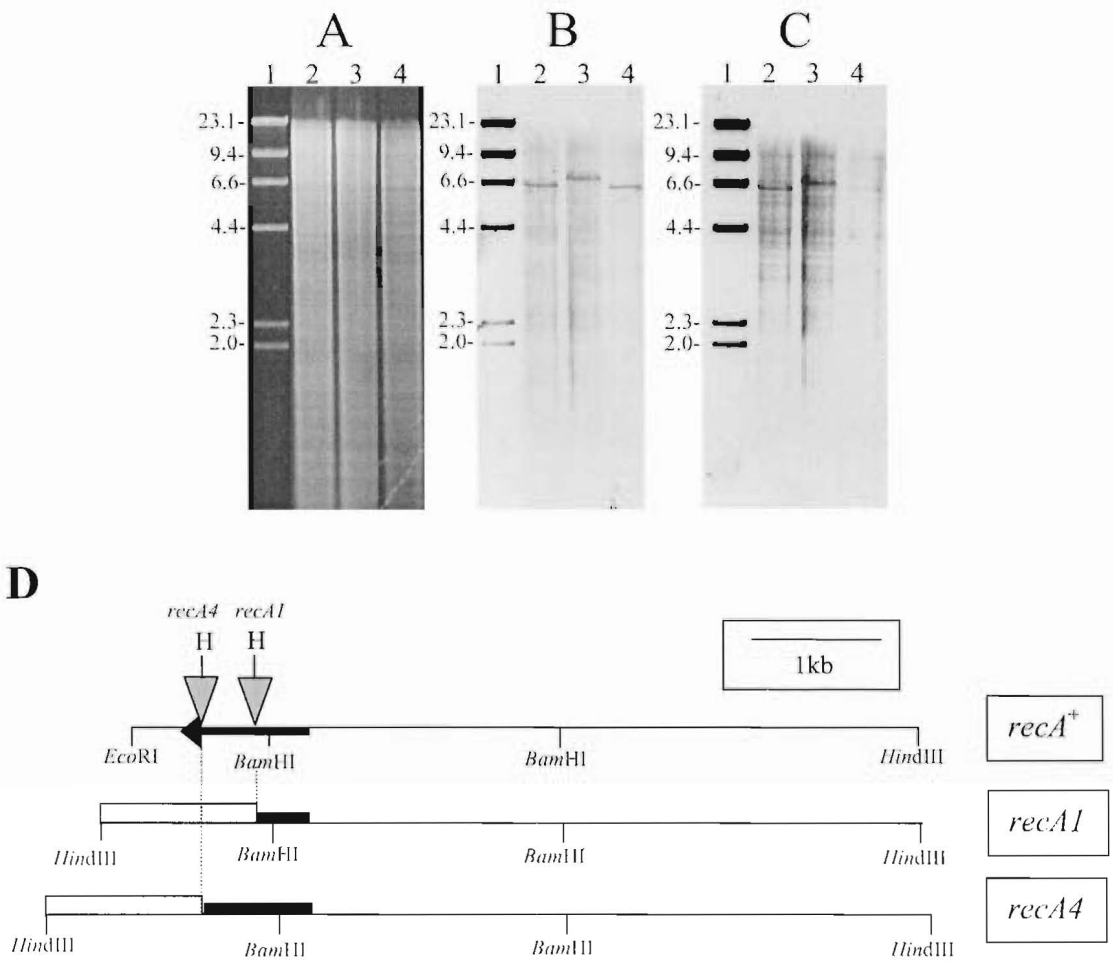


Figure 3.2. Southern hybridisation demonstrating introduction of *recA1* and *recA4* into the genome of PA147-2. In all panels: lane 1, lambda *Hin*dIII standard; lane 2, PA147-2*recA1*; lane 3, PA147-2*recA4*; lane 4, PA147-2. Genomic DNA was digested with *Eco*RI and *Hin*dIII. **A.** Agarose gel showing separation of genomic DNA. **B.** Hybridisation with 6kb *Eco*RI/*Hin*dIII *recA* probe. The fact that the probe hybridises with larger fragments in PA147-2*recA1* and PA147-2*recA4* than PA147-2 indicates incorporation of the DNA with mutations. **C.** Hybridisation with Km^R gene probe from miniTn*10* shows the presence of the transposon. **D.** The restriction fragments that hybridise with the *recA* probe. Because of the *Hin*dIII site in miniTn*10*, the fragments hybridising in the *recA* mutant lanes are *Hin*dIII fragments of different sizes. The grey boxes in **D.** indicate miniTn*10* DNA, the bold horizontal arrow indicates the position and orientation of *recA*, and the bold horizontal lines represent *recA* sequences.

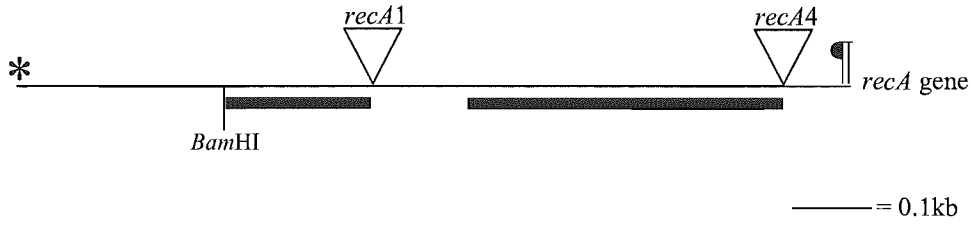


Figure 3.3. Diagrammatic representation of *recA* of PA147-2. Indicates the approximate position of the miniTn*10* insertions in PA147-2*recA*1 and PA147-2*recA*4. The bold horizontal lines represent regions sequenced from the ends of the transposons. * indicates the start codon position, and ¶ indicates the termination codon position.

3.3.4 Stability of cosmids in PA147-2 and PA147-2*recA*::miniTn*10* mutants.

Since the original goal was the creation of a system in which cosmids could be used to complement antifungal mutants *in trans*, the stability of a cosmid in PA147-2 and the *recA* mutant derivatives PA147-2*recA1* and PA147-2*recA4* was examined. The cosmid pPS2122 is a pLAFR3-based cosmid thought to contain at least one gene of importance in antifungal activity and has been shown to restore antifungal activity to *P. aureofaciens* PA138, a Tn*5* mutant of PA147-2, by allele exchange (See chapter 5). However, attempts at complementation *in trans* revealed that pPS2122 was unstable in PA138 if selection for the cosmid was not maintained. Cosmid pPS2122 was introduced to PA147-2, PA147-2*recA1*, and PA147-2*recA4* by conjugation, and maintenance of pPS2122 was tested by growing transconjugants in 10mL LB cultures without selection for the cosmid. Samples of the culture were taken periodically, serially diluted, and plated on LB agar supplemented with Rf , and LB agar plus Rf and Tc. Total colony forming unit counts on Rf selection reflect the total number of culturable cells, whereas only those maintaining the cosmid could grow in the presence of Tc. The results indicate that pPS2122 was considerably more stable in PA147-2*recA1* than in the wildtype (Figure 3.4), whereas there was little difference in stability between PA147-2*recA4* and PA147-2 (data not shown).

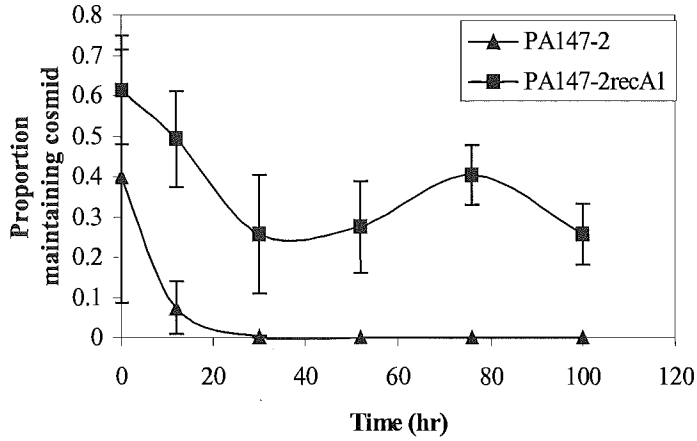


Figure 3.4. Stability of cosmid pPS2122 in PA147-2, and PA147-2*recA1*. The proportion of cells with the cosmid was determined by dividing the tetracycline (Tc) resistant cfu.mL^{-1} by the total cfu.mL^{-1} at each given time point. Tc resistance is conferred by the cosmid, and hence Tc sensitive colonies arise from cells that have lost the cosmid. Data presented are the average of three independent experiments and are shown with the standard error.

The observation that the stability of a pLAFR3 based cosmid is increased in PA147-2*recA1* but not in PA147-2*recA4* is an interesting finding given that very few studies on *recA* have reported similar effects on plasmid stability. An analysis of the archael *recA* analog, *radA*, indicates that *radA* is involved in the maintenance or replication of some halobacterial plasmids in *Haloferax volcanii* (Woods & Dyall-Smith, 1997), while a recent report suggests that plasmid stability can be reduced in *recA* mutants of *E. coli* (Debbia, 1992). These studies demonstrate reduced maintenance of plasmids in *recA/radA* mutants. In contrast, the cosmid pPS2122 shows increased stability in PA147-2*recA1*, but not in PA147-2*recA4*. The transposon insertion points in these mutants have been mapped to near the middle of *recA* (PA147-2*recA1*) and toward the 3' end (PA147-2*recA4*) (Figure 3.3). These data suggest that RecA is involved in the instability of pPS2122 in PA147-2, and that the insertion in PA147-2*recA1* reduces this instability effect. It is possible that the domain of RecA responsible for the plasmid instability is located nearer to the N-terminus of the protein than the insertion in PA147-2*recA4*, and thus it is still expressed in the *recA4* mutant. In PA147-2*recA1*, the insertion point is near the middle of the gene, which appears to be sufficient to abolish the instability phenotype associated with pPS2122 in PA147-2. In this case it is tempting to suggest that the role of RecA in plasmid instability is genetically separable from its role in DNA repair since both *recA* strains show increased sensitivity to DNA-damaging agents, but only one has a change in plasmid stability.

Regardless of the mechanistic basis for the altered cosmid stability in the *recA1* background, the observation of increased stability justified the approach taken and the further work required to create *recA* strains of Tn*5* mutants.

3.3.5 Using pPRAM1 to introduce the *recA*::miniTn*10* allele into Tn*5* mutants of PA147-2.

Because of the success in generating PA147-2 *recA* strains, and the apparent stability of cosmids in the *recA1* background, an attempt was made to generate *recA* mutations in the antifungal-defective mutants of PA147-2. The approach was similar to the construction of PA147-2*recA1*, except that it was not possible to enrich for candidate recombinants using Km^R since the antifungal mutants already express Km^R from Tn*5*. Instead, after incubating the Tn*5* mutant containing pPRAM1 for ten hours without selection, bacteria were simply plated on LB plus Rf. Colonies were then screened for Tc^S to indicate the loss of the plasmid, and MMS^S, to indicate inheritance of the *recA*::Tn*10* allele. Despite screening more than 5000 candidates from multiple independent experiments for the desired phenotypes, no candidate recombinants could be identified for either PA138 or PA109. This could be due to the inability to enrich for candidates, or recombination between the Km^R genes of Tn*5* and miniTn*10* being favoured. Experimental evidence to explain the failure to create *recA* mutants in the Tn*5* mutant strains was not sought. Rather, it seemed more appropriate to modify the system to create defined *recA* mutations.

3.3.6 Construction of partial *recA* deletions in PA147-2 and its mutant derivatives.

DNA sequencing of recA from PA147-2.

Although inactivation of *recA* with pPRAM1 in PA147-2 was successful, the success could not be reproduced when using the Tn*5* mutants of PA147-2. This was problematic since the objective was to create a *recA* mutation in the antifungal mutants of PA147-2. To be useful, the system had to be simple, relatively rapid, and reliable. Since this was not the case with the use of pPRAM1 in the Tn*5* mutants, an alternative was sought. The DNA sequence of *recA* from PA147-2 was determined as a starting point for designing a strategy to delete *recA* in *P. aureofaciens*. This was because knowledge of the sequence could reveal the precise location of useful restriction sites, which could enable the design of a simple method for deleting some (or all) of the gene. It was hoped that construction of a

deletion system would circumvent the difficulties encountered when trying to introduce the *recA1* allele into Tn*5* mutants. Of particular interest was the location of the left-most *Bam*HI site (Figure 3.5) with respect to *recA*.

Previous mutagenesis of pREC1 and pREC6g using miniTn*10* allowed the approximate location of *recA* to be determined. Using this information, a subcloning and sequencing strategy was devised (Figure 3.5). The inserts within the resulting clones, pRECEB, pRECB, and pRECBH were sequenced using T7 and T3 primers.

The DNA sequence of *recA* from PA147-2 is shown in appendix 4, along with the alignment with *recA* from *P. fluorescens*. The sequence revealed that *recA* is encoded right to left relative to the illustration in Figure 3.5, and that the leftmost *Bam*HI restriction site shown in Figure 3.5 is within *recA*. *Bam*HI cuts at position 298 in the gene. The location of one *Bam*HI site within *recA* suggested that deletion of the approximately 2.4kb *Bam*HI fragment from pREC6g would remove part of *recA*. Furthermore, if this proved to be the case it would be possible to introduce the deletion into the genome of PA147-2 and its mutant derivatives by allele exchange, resulting in strains unable to produce RecA.

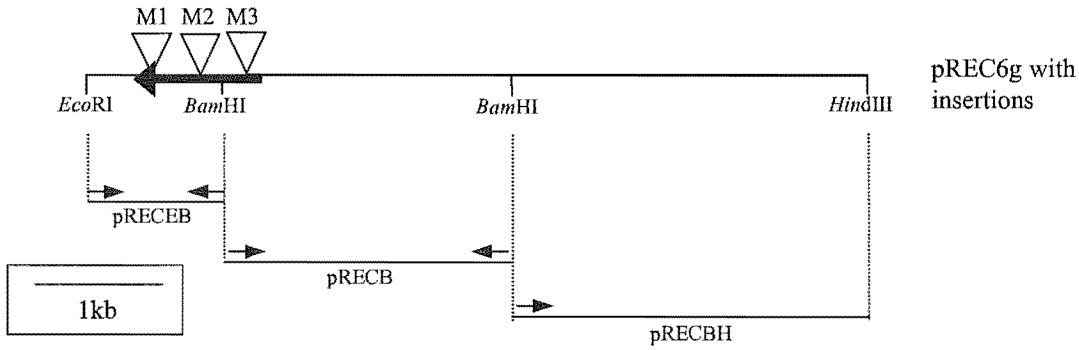


Figure 3.5. pREC6g showing the three miniTn10 insertions. Insertion points in pREC6M1, pREC6M2, pREC6M3 are indicated by triangles. The direction and approximate location of *recA* is indicated by the bold arrow in pREC6g. Also shown are the subclones pRECEB, pRECB, and pRECBH, which were used to determine the sequence of *recA*. Arrows above the subclones indicate the directions of DNA sequencing.

Construction of a recA deletion system.

The plasmid pREC6g is stable in PA147-2. In order to use allele exchange for mutagenesis of *recA*, a poorly maintained vector is desirable. For this reason, the clone pREC6L was used in further *recA* constructions. A 2.4kb *Bam*HI fragment was deleted from pREC6L by digesting the plasmid with *Bam*HI, and then religating the digested DNA without the internal *Bam*HI fragment. The removal of this *Bam*HI fragment resulted in the creation of the plasmid pREC6LΔB (Figure 3.6). To confirm that the deletion had rendered pREC6LΔB unable to express RecA, DH5α(pREC6LΔB) was streaked onto LB agar supplemented with MMS. Whilst the parental plasmid (pREC6L) conferred MMS resistance upon DH5α, DHα (bearing pREC6LΔB was unable to grow in the presence of MMS (data not shown). Based upon these results, it was reasonable to conclude that part of the *recA* gene had been deleted, and this deletion was sufficient to abolish RecA-dependent functions.

Using pREC6LΔB to create recA strains.

pREC6LΔB was introduced into PA147-2 by conjugation, using *E. coli* S17-1 as the donor strain. Transconjugants were pooled and allowed to grow without selection for ten hours, after which the culture was ten-fold serially diluted and plated onto LB containing Rf. Individual colonies that arose were tested for Tc^S (to demonstrate the loss of the plasmid) and MMS^S to show mutagenesis of *recA* by allele exchange. Greater than 90% of colonies tested had lost the plasmid, but despite several attempts, no MMS^S strains were recovered by this technique.

The lack of success using pREC6LΔB to generate a deletion mutation of *recA* in PA147-2 could be due to two possibilities. Firstly, deleting the *Bam*HI fragment may be insufficient to generate MMS^S strains of PA147-2. This seemed unlikely given the MMS^S phenotype of DH5α bearing pREC6LΔB. The second possibility is that the rate of recombination was not sufficiently high to allow the recovery of the desired strains by screening a small minority of the population (e.g. 1000 colonies from a culture containing $1x10^9$ $cfu.mL^{-1}$). To examine the latter suggestion, a *Bam*HI fragment of DNA encoding Gm^R was cloned into the *Bam*HI site of pREC6LΔB, resulting in the plasmid pREC6LΔBg (Figure 3.6).

It was thought that by introducing a selectable marker it might be possible to enrich the population to be tested by selecting those that still possess Gm^R, as was the case when using pPRAM1 to create PA147-2*recA1*. Only those strains that had either retained the plasmid or were recombinants would be Gm^R, thus eliminating those that had simply lost the plasmid. pREC6LΔBg was transferred to PA147-2 by conjugation, and transconjugants were selected on the basis of Gm^R. Transconjugants were pooled and grown in ten mL LB without selection. Ten-fold serially diluted cultures were plated on LB supplemented with Rf and Gm, and single colonies were used to test for MMS^S and loss of the plasmid (Tc^S). In contrast to the results with pREC6LΔB, using pREC6LΔBg resulted in approximately 25% MMS^S strains. These data suggest that the loss of the plasmid is a frequent event, and that enrichment is necessary for easy recovery of recombinants. Without the enrichment step, most colonies that grow probably represent bacteria that have lost the plasmid before recombination took place, but by enriching with a marker these strains are eliminated from consideration. The utility of the technique was then demonstrated by using it to create *recA* deletions in the Tn*5* mutants PA109, PA138, and PAH26. The use of pREC6LΔBg for creation of *recA* strains was successful, and relatively straight forward, and thus should be applicable to future studies.

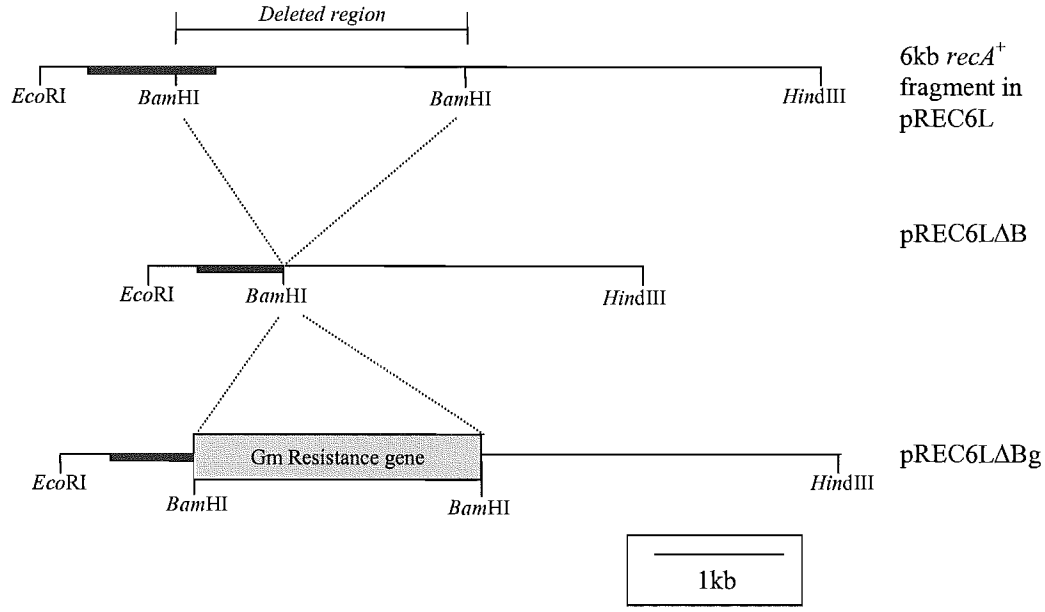


Figure 3.6. Diagrammatic representation of the clones pREC6L, pREC6LΔB, and pREC6LΔBg. The approximate location of *recA* is shown by the bold horizontal line. The reduced length of the bold line indicates that the start of *recA* has been deleted in the ΔB clones.

Confirmation of allele exchange by Southern hybridisation.

Southern hybridisation was used to confirm that the Gm^R MMS^S colonies were indeed due to allele exchange between the wildtype *recA* gene and the Gm^R deletion construct. Total DNA of wildtype PA147-2 and the putative *recA* mutants (Gm^R, MMS^S) of PA147-2, PA109, PA138 and PAH26 (designated PA147-2*recΔ*, PA109*recΔ*, PA138*recΔ*, and PAH26*recΔ* respectively) was digested with *Eco*RI and *Hin*dIII, and separated by electrophoresis. Following transfer to Hybond N+ nylon membrane, the DNA was probed with the 6kb *Eco*RI/*Hin*dIII fragment from pREC6g. The predicted result was that in PA147-2 (*recA+*) a 6kb fragment would hybridise with the probe while in the *recA* mutants a newly introduced *Eco*RI site within the Gm^R gene would result in an approximately 5kb fragment of DNA hybridising with the probe. In addition, there would be no hybridisation to a 6kb fragment in the mutants, which would be sufficient to demonstrate replacement of the 6kb region with that containing Gm^R. The result of the Southern hybridisation is shown in Figure 3.7. All four putative *recA* mutants show the hybridisation pattern that was anticipated for *recA* mutants created using pREC6LΔBg, thus confirming that they are genuine recombinants.

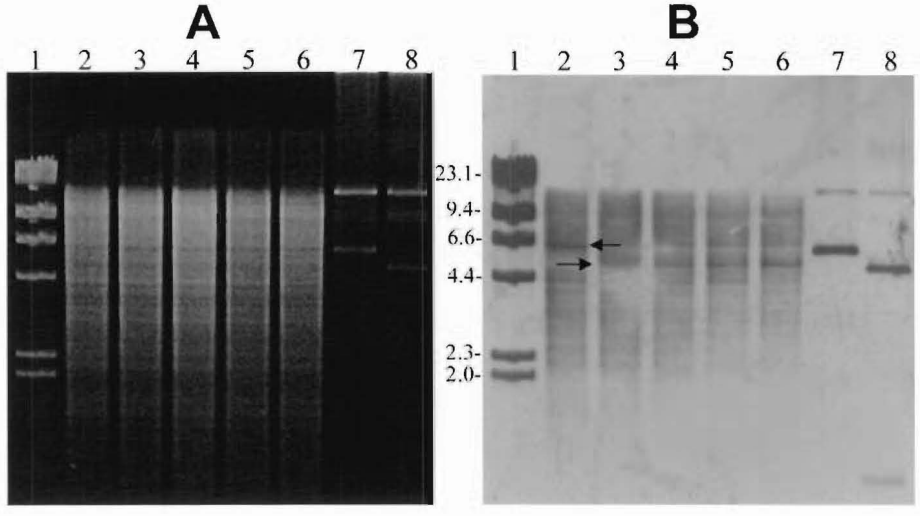


Figure 3.7. Southern hybridisation showing introduction of REC6ΔBg into *P. aureofaciens* strains. In both panels, Lane 1, lambda *Hin*dIII standard; lane 2, PA147-2; lane 3, PA147-2*rec*ΔBg; lane 4, PA109*rec*ΔBg; lane 5, PA138*rec*ΔBg; lane 6, PAH26*rec*ΔBg; lane 7, pREC6L; lane 8, pREC6LΔBg. All digests apart from the standard were *Eco*RI/*Hin*dIII. **A.** Separation of digested DNA by agarose gel electrophoresis. **B.** Southern hybridisation using the 6kb *recA*$^+$ region from pREC6g as a probe. Hybridisation with a 6kb fragment in lanes 2 and 7 shows the presence of the wildtype 6kb *recA* region, whereas hybridisation with a 5kb fragment indicates the presence of REC6ΔBg in the *recA* deletion strains. Arrows indicate the position of hybridised DNA.

In summary, the results presented in this chapter demonstrate that the *recA* gene of PA147-2 is important in the instability of pLAFR3-based plasmids, and that mutating *recA* increases the stability of a pLAFR3-based cosmid. A system for the relatively easy construction of partial *recA* deletions has been developed, and this system has been used to create *recA*$^-$ derivatives of the antifungal minus strains PA109, PA138, and PAH26. In addition to its use in the present study, this system will be of value for the genetic analysis of PA147-2 in future studies.

Chapter 4

Characterisation of antifungal mutants

4.1 Introduction.

During a previous study (Carruthers, 1994) a collection of *P. aureofaciens* isolates defective in their ability to inhibit fungal growth was created by screening a pool of random Tn*5*-generated mutants. Characterisation of some of these mutants is discussed in detail in chapter 1. Briefly, Carruthers showed that two auxotrophs, PAF30 and PAF35, require either all three aromatic amino acids or arginine and uracil respectively to grow. DNA sequence indicated that they have insertions in *aroB* (PAF30) and *carA* (PAF35). Analysis of the mutant PA109 showed that the insertion was in a 16kb *Eco*RI region, and saturation mutagenesis of this region led to the suggestion that it encoded a number of genes involved in production of an antifungal compound(s). Preliminary sequence data indicated that the insertion in PA109 interrupted a putative two-component regulatory gene. Finally, the importance of this genetic region for biocontrol was shown in glasshouse trials by comparing the ability of the wildtype and the mutant PA109 to suppress *Phytophthora* rot of asparagus (Carruthers et al., 1995).

Despite the work outlined above, only a fraction of possible information had been gained from the collection of mutants. Three objectives were defined to significantly improve the understanding of fungal inhibition by PA147-2, and would focus future research efforts on PA147-2. These objectives and their rationale are outlined below.

4.1.1 Objective 1 – Crossfeeding studies.

WHAT IS CROSSFEEDING?

The ability of mutants to complement each other by "crossfeeding" has the potential to demonstrate that different mutants are defective for separate steps in the same biosynthetic pathway. A well-known example of this is the demonstration that pigment production by various pigment mutants of *S. marcescens* can be restored when combinations of mutants are cultured in close proximity to each other. Production of diffusible biosynthetic

intermediates by one mutant compensates for a second mutant's inability to produce the intermediate, thus alleviating the "block" in the biosynthetic pathway (Morrison, 1966). A similar phenomenon occurs when antibiotic-deficient mutants of *Erwinia herbicola* Eh1087 are cultured together on plates. Each mutant alone is incapable of inhibiting the growth of a bacterial lawn. However, when mutants are mixed and plated onto a bacterial lawn, certain combinations inhibit the growth of the lawn. As with the *S. marcescens* example, this is thought to be due to the production of diffusible biosynthetic intermediates by *E. herbicola* (S. Giddens, Pers. Comm.).

DO ANTIFUNGAL MUTANTS CROSS FEED?

An observation that some antifungal mutants of PA147-2 could cross-feed would provide insight into the biosynthesis of the antifungal compound and allow the mutants to be separated into classes based upon crossfeeding results. Conversely, if the mutants were unable to cross-feed, the results could suggest that these mutants have Tn*5* insertions in genes making up more than the biosynthetic pathway, and that the interrupted genes have functions other than modification of precursor compounds. It is important to note that no definitive conclusion can be reached based entirely on negative cross-feeding results since it is also possible that biosynthetic intermediates are not diffusible. However, the possibility that valuable insights could be gained made cross-feeding experiments a logical undertaking.

4.1.2 Objective 2 – Genomic distribution of antifungal genes.

While crossfeeding experiments were intended to give some indirect biochemical information regarding the relationship between the antifungal mutants, they would provide no physical information regarding the organisation of the genes required for antifungal activity. Therefore, the second objective of the preliminary analysis was to establish the physical relationship among the genes that are required for antifungal activity. Since macro-restriction enzyme profiling techniques have previously been shown to be useful in genome analysis and comparison in *Pseudomonas* spp. (Rainey & Bailey, 1996; Smith et al., 1995), field inversion gel electrophoresis (FIGE) and Southern hybridisation were chosen to separate and analyse large restriction fragments from PA147-2 and its mutant derivatives.

The genomic distribution of genes required for the production of antifungal metabolites by PA147-2 was of interest for two reasons. Firstly, from a practical point of view, the organisation of the genes would, to some extent, dictate the way in which the molecular basis for antifungal activity was studied. If the genes were found in close proximity to each other it could be feasible to study them together as an "antifungal cluster". However, a wide distribution would necessitate an approach in which the genes were examined separately. The second reason for examining the distribution of genes is more fundamental, and related to the origin of the antifungal phenotype. Widely distributed genes would lead to the suggestion that the phenotype is not encoded by genes on a horizontally mobile element (such as a plasmid or pathogenicity island), but rather the phenotype is a consequence of secondary metabolism.

4.1.3 Objective 3 – DNA sequencing of transposon insertion points.

A logical objective was to sequence the DNA flanking the transposon insertion points in the mutants, and subject the resulting DNA sequences to similarity searches using BLAST (Altschul et al., 1990; Altschul et al., 1997). Given the vast number of DNA sequences found in the public databases (e.g. Genbank, EMBL), it was possible that putative homologues could be found, and predictions of functional roles in antifungal activity could then be made and tested. It is important to note that while the identification of sequences that were unmatched with any in the databases would be interesting, such a result would not provide any new information regarding antifungal activity.

4.2 Results.

4.2.1 Cross feeding.

CROSS-FEEDING ANTIFUNGAL MUTANTS.

Although PA147-2 consistently inhibited the growth of the fungal colony, no mutant or combination of mutants showed antifungal activity. Thus, the mutants in the collection either do not represent a series of disruptions to a single pathway, or the production of antifungal metabolites by PA147-2 is not characterised by the synthesis of diffusible intermediate compounds.

4.2.2 Genomic distribution of "antifungal genes".

To examine the distribution of genes involved in antifungal activity, field inversion gel electrophoresis (FIGE) was used to separate restriction fragments produced by *Spe*I digestion of PA147-2 and the Tn*5*-generated mutants. DNA was subsequently immobilised on a nylon membrane for Southern hybridisation with a Tn*5*-specific probe. In this way it was possible to examine the distribution of Tn*5* within the antifungal mutants, and thus infer the distribution of genes important in antifungal activity. The FIGE gel and subsequent Southern hybridisation are shown in Figure 4.1. DNA from the wildtype PA147-2 did not hybridise with the probe, verifying the specificity of the probe for Tn*5*. A single restriction fragment hybridised with the probe in each lane containing DNA from antifungal mutants, confirming that each mutant phenotype was the result of a single insertion. The hybridisation pattern shows that the Tn*5* insertions are not found within a single region of the genome, but are spread widely.

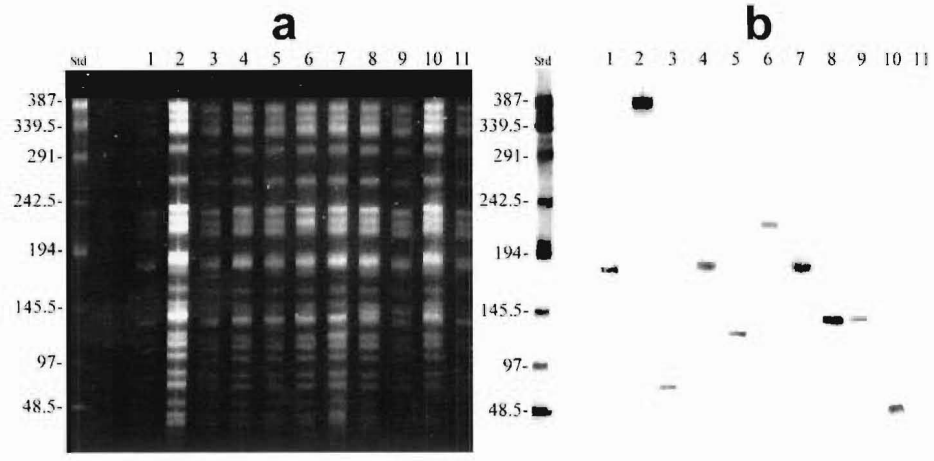


Figure 4.1. Genomic distribution of antifungal genes. **a**. FIGE of *Spe*I digested PA147-2 and the Tn*5*-generated mutants. **b.** Southern hybridisation of DNA fragments separated by FIGE and probed with a Tn*5*-specific probe. Lanes for panels **a.** and **b.** Std= Biorad pulsed-field standard lambda concatemer. 1= PA1. 2= PA138. 3= PAA3. 4= PA109. 5= PAH26. 6= PAE21. 7=PA111. 8= PAF30. 9= PAF35. 10= PAI17. 11=PA147-2. All *P. aureofaciens* isolates are digested with *Spe*I.

4.2.3 DNA sequencing.

DNA sequencing was carried out as outlined in chapter 2, using the clones described in section 2.7 and a primer that anneals to IS*50* of Tn*5* (appendix 3). The results are

summarised in Table 4.1 and discussed below with respect to antifungal activity. Sequences are shown in appendix 4.

Table 4.1. Results of database searches for sequences similar to those derived from the antifungal mutants.

Mutant	Program[a]	Similarity?[b]	Score	Accession number
PA1	Blastn	Nothing significant	N/A	N/A
	Blastx	Nothing significant[c]	N/A	N/A
PA109	Blastn	Nothing significant	N/A	N/A
	Blastx	sensor/response regulator hybrid (*P. aeruginosa*)	$1e^{-168}$	AAG05000.1
PA138	Blastn	Nothing significant	N/A	N/A
	Blastx	GstR (*Bradyrhizobium japonicum*)	$2e^{-44}$	AAC17939
PA168	Blastn	Nothing significant	N/A	N/A
	Blastx	Nothing significant	N/A	N/A
PAA3	Blastn	Nothing significant	N/A	N/A
	Blastx	Nothing significant	N/A	N/A
PAH26	Blastn	*pstA* (*P. putida*)	$1e^{-138}$	AB017356
	Blastx	PstA (*P.aeruginosa*)	$9e^{-27}$	AAG08752
PAE21	Blastn	Nothing significant	N/A	N/A
	Blastx	Gene for galactosyl transferase (*P. aeruginosa*)	$9e^{-18}$	AAF23992
PAI17	Blastn	Nothing significant	N/A	N/A
	Blastx	Nothing significant	N/A	N/A
PAI75	Blastn	3' end of gene for alginate lyase (*Halomonas marina*)	$1e^{-13}$	AB018795.1
	Blastx	Nothing significant	N/A	N/A
PAI95	Blastn	Nothing significant	N/A	N/A
	Blastx	Putative multidrug efflux pump protein RagC (*B. japonicum*)	$5e^{-39}$	T31341

Notes. [a]. www.ncbi.nlm.nih.gov/blast. [b] Similar sequences at nucleotide and predicted translation product levels. Significance was arbitrarily cut-off at E-scores of less than e^{-10}. [c] The predicted translation product from a 470bp sequence starting 49bp away from the insertion point is similar to a predicted protein from *P. aeruginosa* (AE004589)

4.3 Discussion.

4.3.1 Crossfeeding.

The results of the crossfeeding experiments show that no members of the existing collection of antifungal mutants have the ability to crossfeed. This suggests that either the mutations are not affecting a single biosynthetic pathway, or that none of the mutants produce diffusible pathway intermediates that can restore activity to another mutant.

4.3.2 FIGE.

The Tn*5* insertions that result in an antifungal-deficient phenotype in PA147-2 appear to be widely spread throughout the genome, as shown by the hybridisation of the Tn*5* probe to seven different sized fragments, ranging from approximately 50 to 400kb, from the ten mutants tested. This suggests that at least some of the genes for antifungal activity are not clustered, which is consistent with the theory that antifungal activity by PA147-2 is an example of secondary metabolism. The minimum size of the DNA region that could contain all the genes identified is approximately 560kb, indicating that at least some of the genes for antifungal activity are not found on a horizontally mobile element. This assessment of distance is likely to be an underestimate, since it assumes that the insertions on 380kb and 220kb regions are at one end of the fragments, that these fragments flank all of the other *Spe*I fragments that have insertions, and that fragments with insertions are not separated by any without insertions. Thus, the total of 560kb is the sum of the sizes of all fragments that have insertions, apart from the 380 and 220kb fragments.

The Southern blot data obtained from the FIGE-separated *Spe*I fragments shows that the three mutants PA109, PA111, and PA1 all have insertions in an approximately 180kb *Spe*I fragment. A previous study (Carruthers et al., 1994) had shown that these three mutants all have insertions within 3kb of each other in the same 16kb *Eco*RI region. *Spe*I cuts rarely in the PA147-2 genome, and digestion of the 16kb *Eco*RI region showed that it did not cut between the Tn*5* insertions in PA109, PA1, and PA111. Thus, the three mutants were expected to have insertions on the same *Spe*I fragment, allowing them to serve as internal quality controls for this experiment.

The Tn*5* insertions in the auxotrophic mutants PAF30 and PAF35 both appear to be on a *Spe*I fragment of approximately 140kb in size. It is therefore possible that the two genes interrupted in these mutants are located within 140kb of each other (although the possibility of similarly sized fragments co-migrating has not been ruled out). This proximity is probably not suggestive of an antifungal biosynthetic cluster because the genome sequence of *P. aeruginosa* shows these genes are within 300kb in *P. aeruginosa.*. In a previous study, preliminary DNA sequence data was obtained from the region flanking the insertions in these mutants (Carruthers, 1994). The sequences predict that the gene *aroB* (encoding 3-dehydroquinate synthase) is interrupted in PAF30, and PAF35 has an insertion in *carA*, which codes for the small subunit of carbamoylphosphate synthetase. These enzymes are required for aromatic amino acid biosynthesis and pyrimidine and arginine biosynthesis respectively, which is consistent with the auxotrophic requirements of the mutants.

4.3.3 DNA sequencing.

REGULATORY GENES.

The mutants PA109 and PA138 both appear to have Tn*5* insertions in genes that encode global regulators.

In the case of PA109, the mutation is in a gene that encodes a two-component regulator, in agreement with previous findings (Carruthers, 1994). Two-component regulators are widespread in microbes, controlling systems such as extracellular proteolytic activity of *Staphylococcus aureus* (Fournier & Hooper, 2000), capsule production by *Streptococcus pneumoniae* (Levin & Wessels, 1998), and secondary metabolite production by *P. fluorescens* (Corbell & Loper, 1995). Given the involvement of two-component regulators in numerous cellular processes, and particularly in expression of virulence factors and secondary metabolites, it is not surprising to find that such a system plays a role in expression of antifungal metabolites in *P. aureofaciens* PA147-2.

The Tn*5* insertion in PA138 occurs in a sequence whose predicted translation product has similarity to LysR-type transcriptional regulators. Like the two-component system, members of the LysR family have been shown to be involved in the regulation of a variety of processes. Examples include regulation of β-lactamase in *P. aeruginosa* (Lodge et al.,

1993) and *Streptomyces cacaoi* (Magdalena et al., 1997), catabolism of phenolic compounds by *Agrobacterium tumefaciens* (Parke, 1996), and regulation of the production of the antifungal compound pyoluteorin by *P. fluorescens* (Nowak-Thompson et al., 1999).

The *P. aureofaciens* mutant PA109 was of interest because the results of Carruthers (1994) indicated that the two-component regulator gene disrupted in PA109 was located within an antifungal gene cluster. PA138 was chosen for further study because at the time the initial sequence was obtained, there were no reports of LysR-type transcriptional regulators involved in antifungal activity. The mutants PA109 and PA138 are the subject of chapter 5.

MUTANT PA1.

The Tn*5* insertion point in PA1 was previously shown to map 2.1kb away from the insertion in PA109 (Carruthers 1994; Carruthers et al., 1994). However, no DNA sequence covering the insertion point was obtained in those studies, so the question of whether or not PA1 had an insertion in the same gene as PA109 remained unanswered. The DNA sequence data generated in the present study suggests that PA1 has an insertion 43bp downstream of the putative initiation codon of the two-component regulator that is disrupted in PA109, and is 49bp downstream of an ORF of unknown function. The position of the insertion derived from the sequence data indicates it is most likely that the Tn*5* has interrupted the two-component regulator gene, thus preventing expression of the gene product. The lack of sequence similarity shown in Table 4.1 can be explained by the fact that the DNA sequence from clone pCM1 reads toward the start of the two-component gene, and finishes upstream of the gene.

PAH26.

PAH26 has a Tn*5* insertion in a sequence that is predicted to encode the gene *pstA*, based upon similarity to *pstA* from *P. aeruginosa* and *P. putida*. In other bacterial species, PstA forms part of the membrane-spanning portion of the phosphate specific transport complex, which is an ABC transporter required for high affinity uptake of inorganic phosphate during phosphate limitation (Cox et al., 1988). Thus, PstA is important during phosphate starvation. In addition to its role in phosphate transport, an intact Pst complex is required

for the repression of the phosphate (Pho) regulon, which is expressed during phosphate limitation (Wanner, 1996). Null mutations to *pstA* would be predicted to result in an inability to rapidly uptake inorganic phosphate under phosphate starvation conditions, and constitutive derepression of the Pho regulon. The fact that a *pstA* mutation renders *P. aureofaciens* PA147-2 antifungal minus leads to the following hypotheses: 1. phosphate uptake is required for antifungal activity; 2. expression of the Pho regulon represses antifungal activity; 3. Pst has roles in PA147-2 antifungal expression in addition to those characterised in other organisms. These hypotheses are tested in relation to the mutant PAH26 in chapter 6.

PAI95.

The predicted translation product from the DNA sequence derived from the Tn*5* insertion point in PAI95 shows a high degree of similarity to RagC from *Bradyrhizobium japonicum* (Krummenacher & Narberhaus, 2000), and to a number of efflux systems and drug transport proteins. Thus, PAI95 is predicted to have a mutation in a cytoplasmic membrane protein that functions as a pump from the cytoplasm to the periplasm. With respect to antifungal activity, there are two obvious roles that such a pump could potentially play. Firstly, a pump might be required for the transport of one or more antifungal metabolites from the cytoplasm to the extracellular environment. Proteins with similar sequences have been implicated in the efflux of toluene (Ramos et al., 1998), and resistance to a number of dyes, detergents and antibiotics by pumping them out of the cell (Ma et al., 1993). A second possible role is environmental sensing. It is conceivable that a pump could allow *P. aureofaciens* PA147-2 to sense the presence of a fungal competitor, perhaps by the uptake of a particular molecule from its environment. Such a sensing system would allow PA147-2 to specifically respond to the presence of a fungus, rather than synthesising antifungal compounds constitutively.

Although there is no evidence for either of the above suggestions, some indirect evidence indicates that the second possibility is less likely. If PA147-2 uses a membrane channel to detect and respond to fungi, then it is likely that antifungal compounds would not be constitutively expressed. However, it has been possible to prepare a crude extract of antifungal compounds from a culture of PA147-2 that has not been exposed to fungi. This

indicates that fungi are not required to induce synthesis of antifungal metabolites, although it does not rule out fungi inducing an increase in compound synthesis from a "normal" basal level. However, if this was the case, PAI95 would be predicted to produce some antifungal compounds at the basal rate, but this has not been observed. Additionally, an inducer need not be fungal – the possibility exists that something in the culture media used can induce synthesis. Indeed, glucose concentration has been shown to influence production of antifungal compounds by *P. fluorescens* HV37a (James & Gutterson, 1986) and carbon sources affect pyrrolnitrin and pyoluterin production in *P. fluorescens* (Duffy & Defago, 1999). Godfrey (1997) reported that glucose concentration did not influence antifungal activity by PA147-2, and no effect of carbon source has been observed throughout the course of the present study. However, it is important to note that optimisation of synthesis conditions was not an objective of this work, so no explicit examination of media conditions has been made.

The possibility that the mutated gene in PAI95 encodes a transmembrane pump is intriguing, and requires further study. Tn*phoA* mutagenesis would be a straightforward method to examine the membrane topology of the protein, and cell lysis experiments might reveal whether antifungal compounds are being synthesised, but not exported.

PAE21.

Based on similarity searches, the Tn*5* insertion in PAE21 appears to interrupt a sequence whose predicted translation product is similar to a galactosyl transferase enzyme from *P. aeruginosa* serotype O6 (Belanger et al., 1999) and *Vibrio cholerae* O139 (Comstock et al., 1996). Specifically, the translation product is similar to WbpU, which is an α-D-glycosyl transferase in *P. aeruginosa* (O6 serotype). Belanger et al (1999) suggest that WbpU is similar in function to RfbF from *Klebsiella pneumoniae*, which is an initiating galactosyl transferase. It is thought that mutations in *wbpU* would result in loss of B-band O antigen biosynthesis. If this were the case in PAE21, it would be possible to detect the absence of the B-band by comparing LPS of PAE21 with PA147-2 using a tricine-SDS-PAGE system (Lesse et al., 1990).

Exactly how loss of B-band O antigen synthesis affects antifungal activity is unknown. The O antigen is a significant component of the outer membrane, and has been implicated as a virulence factor, enabling pathogens to evade the immune system (Jacques, 1996; Schnaitman & Klena, 1993). In addition, O antigen is thought to have some role in cell-cell interactions between pathogens and hosts, and may be involved with the secretion of extracellular compounds (Wandersman & Letoffe, 1993). In terms of antifungal activity, it seems unlikely that an O antigen is important for protection of the antagonistic bacterium, since the expression of antifungal factors effectively minimises any close physical interaction between bacteria and fungus. It seems more likely that alterations to the cell surface interfere with secretion of the antifungal compound(s) produced by PA147-2, leading to the observed antifungal-deficient phenotype of PAE21.

PAA3, PAI17, PA168, AND PAI75.

The DNA sequences that flank the transposon insertion points in PAA3, PAI17, and PA168 have no significant similarity to any sequences deposited in public databases, at both DNA and predicted translation product level. For these mutants, DNA sequencing has failed to provide any clues about the possible functions of mutated genes, and does not lead to any speculation about why these mutants are unable to inhibit fungal growth. The sequence from PAI75 showed short stretches of similarity to two regions from the 3' end of a gene for alginate lyase in *Halomonas marina*. However, the fact that there was no similarity at the predicted translation product level suggested that the sequences do not encode similar proteins. Examination of the similar sequences shows that one region from the PAI75 sequence is similar to two stretches of DNA from the alginate lyase gene, and these two sequences are adjacent to each other, in opposite directions. It is thus possible that the mRNA from them can form a stem and loop structure, and they may be a termination sequence (Figure 4.2).

a.

```
PAI75: 391  tgatcnttcccacgctccgcgtgggaatgccgccccggacgctccgcgttccatgcc 447
            ||||| ||||||||||||||||||||||||| |||| |||||||||||||| |||||||
H.mar: 1441 tgatcgttcccacgctccgcgtgggaatgcagcccgggacgctccgcgtcccatgcc 1497 (Plus)


PAI75: 392  gatcnttcccacgctccgcgtgggaatgcc 421
            |||| |||||||||||| ||||||||||||
H.mar: 1552 gatcgttcccacgctctgcgtgggaatgcc 1523 (minus)
```

b.

Figure 4.2 Comparison of sequence from PAI75 with sequence from an alginate lyase gene. **a.** Alignment of the sequence from PAI75 with two short sequences from the alginate lyase gene from *Halomonas marina* (H.mar). "Plus" indicates the plus strand, while "minus" indicates the sequence shown is the minus (opposite) strand. **b.** Putative stem and loop structure formed by the alginate lyase sequences. The minus strand sequence was converted to a plus strand for this arrangement.

4.3.4 What does all of this mean?

The DNA sequences and predicted translation products lend themselves to speculation regarding possible roles in fungal inhibition. Since this chapter does not test the functions of the mutated genes, there is no way to know whether the database matches accurately predict the functional nature of these genes. However, regardless of whether the sequences described here actually carry out their predicted functions, one thing is clear – none of the identified sequences show similarity to the biosynthetic genes for common antifungal metabolites such as phenazines, pyrrolnitrin, pyoluteorin, and 2,4-diacetylphloroglucinol. It seems unlikely that the sequences uncovered in this study are of biosynthetic genes; rather, these sequences probably participate primarily in normal cellular activities, and have been recruited into the secondary metabolism over evolutionary time. The fact the mutants do not cross-feed, and that the mutations are spread throughout the genome supports this suggestion because biosynthetic genes for antifungal compounds identified to date tend to be clustered (Bangera & Thomashow, 1999; Hammer et al., 1997; Mavrodi et al., 1998; Nowak-Thompson et al., 1999)

The failure to isolate obvious biosynthetic genes could indicate that the original mutant screen did not cover the whole genome. Alternatively, PA147-2 may not synthesise

antibiotics using gene products that resemble those already known. The isolation of global regulatory mutants deficient for antifungal compound synthesis suggests that there are target genes that encode antibiotic synthesis proteins. The failure to find these could also be explained by a masking effect, where mutants for a single antibiotic are not found due to production of a second compound. Global regulators that prevent synthesis of all compounds would thus be much easier to find, as appears to be the case for PA147-2. Knowledge of the inhibitory compounds produced by PA147-2 would potentially facilitate the development of a system to screen mutants for the loss of individual compound synthesis.

Given the results outlined above, it was decided that the most logical way to examine the molecular basis for fungal inhibition was to investigate mutants individually. Both of the regulatory mutants (PA109 and PA138) were chosen for further study because they might reveal details on the regulatory hierarchy controlling the antifungal phenotype. In addition, it might be possible to develop methods to identify their targets, which could lead to the identification of the biosynthetic genes. These mutants are discussed in more detail in chapter 5. The *pstA* mutant (PAH26) was also selected for further analysis because of the interesting link between "normal" metabolism (phosphate assimilation) and what is presumably an example of secondary metabolism. PAH26 is examined further in chapter 6.

Chapter 5

Global regulators involved in fungal inhibition

5.1 Introduction.

Fluorescent pseudomonads that are of interest in biocontrol produce a range of secondary metabolites that inhibit fungal growth *in vitro* and correlate with disease suppression *in situ* (for example, Keel et al., 1990; Thomashow & Weller, 1988). Well-characterised strains *P. fluorescens* CHA0 and Pf-5 both produce HCN (Kraus & Loper, 1992; Voisard et al., 1989), 2,4-diacetylphloroglucinol (Phl) (Maurhofer et al. 1992; Nowak-Thompson et al., 1994) and pyoluteorin (Howell & Stipanovic, 1980; Maurhofer et al., 1992), and pyrrolnitrin (Howell & Stipanovic, 1979). Several levels of regulation are important for the control of antibiotic production by these and other fluorescent pseudomonads, and individual metabolites are often controlled in part by positive feedback loops (Haas et al., 2000). In addition, the observation of mutants with pleiotrophic defects in antibiotic synthesis revealed that there are global regulators that operate at a level above specific metabolite control, to coordinate the expression of antifungal metabolites.

One global regulatory system is known as the "two-component signal transduction" system, which typically consists of a sensor (histidine protein kinase) and a response regulator (Parkinson & Kofoid, 1992; Stock et al., 1990). For example, the product of *gacA* is a response regulator member of a two-component system that was found to control the synthesis of multiple compounds in *P. fluorescens* CHA0 (Laville et al., 1992), and was subsequently shown to be important for survival of CHA0 in soil (Natsch et al., 1994). The sensor kinase partner of GacA, ApdA (now known as GacS), was independently demonstrated to be essential for antibiotic production by *P. fluorescens* Pf-5 (Corbell & Loper, 1995). Production of HCN, pyrrolnitrin, and chitinase by *P. fluorescens* BL915 is also dependent on the two-component system GacA/S (Gaffney et al., 1994). Furthermore, the stationary phase sigma factor encoded by *rpoS* is required by Pf-5 for pyrrolnitrin production, while pyoluteorin and Phl are over-expressed by *rpoS* mutants of Pf-5 (Sarniguet et al., 1995).

5.1.1 Two-component signal transduction.

Two-component signal transduction systems are involved in many processes that utilise an environmental signal, such as virulence/pathogenesis (Hrabak & Willis, 1992; Huang et al., 1993; Lucas et al., 2000; Rahme et al., 1995), antibiotic synthesis (Chancey et al., 1999; Corbell & Loper, 1995; Gaffney et al., 1994; Laville et al., 1992), response to phosphate limitation (Wanner, 1996), chemotaxis (Hess et al., 1988a; Hess et al., 1988b), osmoregulation (Forst et al., 1989; Igo et al., 1989), and degradation of aromatic hydrocarbons (Coschigano & Young, 1997; Labbe et al., 1997; Velasco et al., 1998). Two-component regulators operate by sensing an environmental cue via a sensor kinase (SK), and subsequently activating gene expression via a response regulator (RR). This system employs auto-phosphorylation of the SK, and subsequent transfer of the phosphate to the RR, upon sensing the appropriate signal. Signal transduction is achieved by the utilisation of specialised modules within the two-component proteins. Sensors generally have a specific input domain which receives the signal, and the transmitter module then passes the signal ("transduces") to the receiver module of the RR via phosphorylation, thus activating the RR. The output domain of the RR is then responsible for activating target genes. Although the transmitter and receiver modules are conserved, the input and output domains are unrelated, allowing specific signals to be acted upon with minimal cross talk between systems (Parkinson & Kofoid, 1992). Auto-phosphorylation of the SK occurs when the SK uses ATP to phosphorylate a highly conserved histidine residue. When a signal is received, this phosphate is transferred to an aspartic acid side chain in the receiver module of the RR (Stock et al., 1990).

5.1.2 LysR-type transcriptional regulators.

LysR-type transcriptional regulators (LTTRs) are perhaps the most common regulatory systems in prokaryotes (Schell, 1993). LTTRs possess some or all of the following characteristics:

1. High degree of similarity at the N-terminus, particularly in a helix-turn-helix domain.
2. Responsive to specific co-inducer molecules.
3. Bind regulated targets with similar structural features at similar positions.
4. Divergently transcribed from a promoter close to that of a regulated target.
5. Show some degree of autoregulation.

LTTRs are often found linked to a target gene, in a divergent arrangement, but are also known to regulate unlinked regulons (Schell, 1993). In keeping with the ubiquity of LTTRs in prokaryotic regulation, they regulate a diverse range of cellular activities. Examples include virulence factors (Clough et al., 1997; Harris et al., 1998), exotoxin production (Hamood et al., 1996), aromatic hydrocarbon catabolism (Laurie & Lloyd-Jones, 1999; Parke, 1996), and antibiotic resistance (Lodge et al., 1993; Magdalena et al., 1997). Despite the large range of targets, the reported involvement of LTTRs in synthesis of antifungal compounds is limited to a single example, in which the pyoluteorin regulator (PltR) specifically regulates pyoluteorin production in *P. fluorescens* Pf-5 (Nowak-Thompson et al., 1999).

MECHANISM OF LTTR ACTION.

In his 1993 review, Schell describes a model for LTTR-mediated gene regulation (Schell, 1993). This model is briefly described here. Most LTTRs, acting as dimers or tetramers, bind a ~15bp partially dyadic sequence, featuring the T-N_{11}-A motif, within the promoter of the target gene. This "recognition site" is generally found near position –65, and because of the divergent promoter arrangement, this site can overlap the LTTR's own promoter. Thus, when regulating the downstream gene, binding can result in the negative autoregulation that characterises some LTTRs. Nucleotides of the dyad are thought to provide for specificity of recognition for binding. In addition to the recognition site, there is an "activation site" downstream (near the –35 sequence), with which LTTRs interact. Interaction with the activation site often requires a co-inducer molecule, and is necessary for transcriptional activation of the target gene. It seems that a co-inducer may alter the LTTR-DNA complex, possibly by inducing DNA bending that would lead to transcription initiation by increasing RNA polymerase affinity. A large number of different co-inducers have been identified, and these are thought to be specific for each regulator. Examples include diaminopimelate, a precursor in lysine biosynthesis, acting as co-inducer for LysR, a regulator in lysine biosynthesis, and cyanate is thought to be the co-inducer for CynR, which is involved in cyanate detoxification. These and other examples are summarised by Schell (1993). One thing that seems evident among known co-inducers is that they tend to be compounds related to the function of the regulator, such as biosynthetic intermediates.

5.1.3 Regulation of antifungal compound synthesis.

The well-characterised antifungal metabolites are regulated on more than one level. They are generally subject to global regulation, in which multiple traits are simultaneously coordinated and controlled, and each appears to be the target of specific regulation. Multilevel regulatory systems presumably allow fine control over phenotypes regulated in this way. Because of the relevance to the work presented in this chapter, details on global regulation of antifungal compound synthesis are provided below. A review of metabolite-specific regulation is given in chapter 1 of this volume.

GLOBAL REGULATION.

In addition to the specific regulation of the production of particular antifungal compounds, the synthesis of antifungal metabolites is also controlled collectively by global regulators (Corbell & Loper, 1995; Gaffney et al., 1994; Laville et al., 1992). The two-component regulators are a prominent example of global regulators, and *gacA/S* in particular has been heavily implicated in the regulation of antibiotic production.

gacA/S.

The two components of the *gacA/S* system were initially identified separately. A mutation in the *gacA* gene (global regulator of antibiotic and cyanide) was found to be the cause of pleiotrophic defects in secondary metabolite production by *P. fluorescens* CHA0 (Laville et al., 1992), while *gacS* (initially called *lemA*, for lesion manifestation), was found to be required for formation of disease lesions on bean plants by *P. syringae* (Hrabak & Willis, 1992). *lemA* was subsequently found to be important for secondary metabolite production by *P. fluorescens* Pf-5 (where it was called *apdA*, for antibiotic production) (Corbell & Loper, 1995), and was finally renamed *gacS* (Kitten et al., 1998). The demonstration that mutations in *gacA* and *gacS* have pleiotrophic effects provided clear evidence that these two genes were involved in global regulation (Corbell & Loper, 1995; Laville et al., 1992). As previously mentioned, the mechanism by which the response regulator (RR) acts is thought to be determined by its output domain (Parkinson & Kofoid, 1992). Evidence suggests that the RR GacA is a transcriptional activator, at least in the case of phenazine

production, in which GacA regulates PhzI production (Chancey et al., 1999). However, in the case of HCN synthesis GacA appears to influence metabolite production via a post-transcriptional mechanism, as demonstrated by the fact that *gacA* mutations had minimal impact on expression of a *hcnA*::*lacZ* transcriptional fusion, but profoundly reduced expression of a translational *hcnA*::*lacZ* fusion (Blumer et al., 1999). This observation was also extended to include expression of AprA, a GacA-regulated metalloprotease. Small changes in the ribosome binding site (RBS) of the GacA-regulated genes in CHA0 were sufficient to repress GacA control. It is interesting to note that the same sequences that were responsive to GacA post-transcriptional regulation were also susceptible to repression by the repressor of secondary metabolism, RsmA. Since RsmA is a global negative regulator, Blumer et al (1999) suggest a model in which GacA increases expression of another regulatory molecule, which can relieve RsmA repression. Of further interest was that the expression of *hcnA* from *P. aeruginosa*, which usually is controlled by a system similar to PhzR/I (which is in turn regulated by GacA), was poorly expressed in CHA0, indicating that GacA mediates control of similar systems in a variety of ways.

Regulation of PCA expression in *P. aureofaciens* 30-84 is controlled by GacA/S in two ways. Firstly, there is direct transcriptional activation of *phzI* (Chancey et al., 1999). However, since addition of *N*-acyl-homoserine lactones did not restore PCA production in 30-84 *gacA* mutants, an additional mechanism for GacA-mediated control must exist (Chancey et al., 1999), and it has been speculated that a post-transcriptional control similar to that described above might be involved (Haas et al., 2000).

GacA is also important in the regulation of Phl production. In a *gacA* mutant of *P. fluorescens* CHA0, expression of a *phl*::*lacZ* fusion was reduced considerably, and the fusion was only minimally responsive to Phl induction (Schnider-Keel et al., 2000). Thus it appears that GacA operates at a level above PhlF, since disruption of *gacA* effectively removes the PhlF-dependent subtleties from the system.

The systems outlined above, and in chapter 1, provide a detailed and complex picture of regulation with respect to secondary metabolite production. There is a clear rationale for the further investigation of antifungal compound regulation, particularly in strains such as

P. aureofaciens PA147-2 that do not appear to make the same range of compounds as the better characterised biocontrol pseudomonads. The sequence data presented in chapter 4 suggested that mutations in two types of regulator lead to abolition of fungal inhibition by PA147-2. It was of interest to examine these mutants in more detail for three reasons. Firstly, the examination of regulatory mutants will add to the growing picture of global regulation of secondary metabolism. Secondly, by investigation of regulatory mutants it might be possible to identify some of the regulated targets, thus revealing additional genes for fungal inhibition. This second reason was particularly compelling given that analysis of Tn*5* mutants had failed to reveal the nature of the antifungal compounds produced by PA147-2. Finally, only one LTTR has been implicated in biocontrol thus far, and it has a very specific function in pyoluteorin synthesis. Investigation of the putative LTTR identified here might provide the first example of a LTTR involved in global regulation of fungal inhibition.

5.2 Analysis of a putative LysR-type transcriptional regulator.

5.2.1 Complementation of the mutation in PA138.

Three cosmids spanning the insertion point in PA138 were introduced into PA138 by conjugation, and the transconjugants were assessed for their ability to inhibit fungal growth. None of the three cosmids was able to restore antifungal activity to PA138, and the same result was observed when a *recA* derivative of PA138 was used. These results suggested that either PA138 had an additional, unknown mutation that contributed to the loss of fungal inhibition, or inserts contained in the cosmids were not complementary to the Tn*5*-disrupted region. To test the latter suggestion, the cosmids were digested with a variety of restriction enzymes, and analysed by Southern hybridisation. Each cosmid had a sequence that hybridised with the insert DNA from the clone pCM138, confirming that the cosmids were genuinely derived from the correct genomic region. To further examine the problem, one cosmid (pPS2122) was used in an allele-exchange experiment, to replace the Tn*5*-mutated region in PA138. PA138(pPS2122) was grown without selection for 24 hours, and diluted to obtain single colonies upon plating. The colonies were tested for Tc^S (indicates loss of cosmid vector) and Km^S (indicating loss of Tn*5*). Two putative recombinants were isolated and tested for antifungal activity. Results from the bioassay confirmed restoration of fungal inhibition, and the allele-exchange was confirmed by Southern hybridisation (Figure 5.1). Therefore, the mutation in PA138 was unable to be complemented *in trans*, but could be complemented by allele-exchange. This result raised the possibility that the mutated gene was dominant over the wildtype, since the presence of the wildtype *in trans* did not restore antifungal activity. This suggestion is examined below.

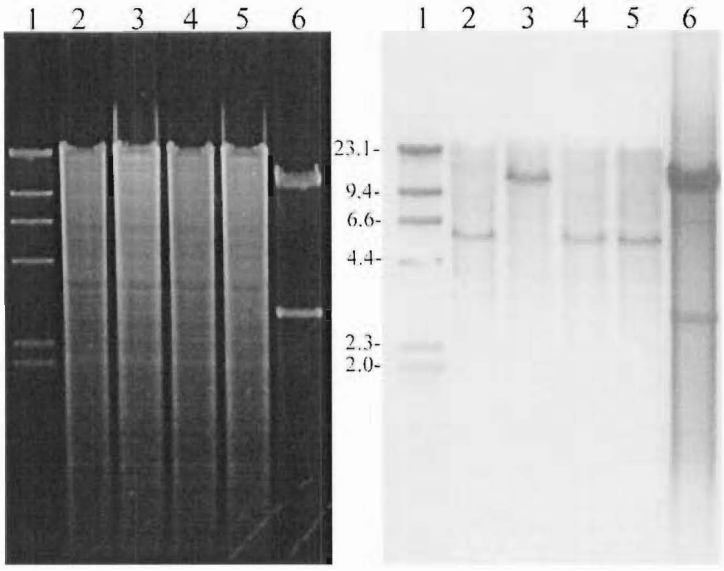


Figure 5.1. Southern analysis of allele-exchange between pPS2122 and the PA138 genome. Lambda DNA digested with *Hin*dIII (lane 1) is shown as a molecular weight standard. DNA from PA147-2 (lane 2), PA138 (lane 3) and two recombinants (lanes 4 and 5) was digested with *Eco*RI and probed with the cloned region from pCM138. pCM138E (lane 6) was included as a control. The Southern was probed with the 4kb *Sal*I fragment cloned in pCM138. Hybridisation of the probe with a fragment of 5.5kb in the recombinants shows a wildtype region of DNA has replaced the mutated region (11.5kb).

5.2.2 Plasmid constructs.

All plasmids constructed in the study of PA138 are detailed in chapter 2, section 2.7.3. Plasmids created in the study of PA109 are described in section 2.7.4 of chapter 2.

5.2.3 Is the defective gene of PA138 dominant over the wildtype?

To test the hypothesis of recessive dominance, the clone pCMsub was introduced into PA147-2 by conjugation. The clone pCMsub is a subclone from pCM138E containing *finR* interrupted by Tn*5*, as it is in PA138. PA147-2(pCMsub) was assessed for its ability to inhibit fungal growth relative to PA147-2 harbouring the vector pME6000. The result, shown in Figure 5.2, demonstrates that when the mutated allele is present *in trans*, PA147-2 is reduced for it ability to inhibit fungal growth. This observation is consistent with the hypothesis that the mutated gene is dominant over the wildtype.

Figure 5.2. Inhibition of *P. megasperma* var. *sojae* by PA147-2 and derivatives. Four colonies (A-D) of PA147-2 containing pCMsub were compared with the wildtype PA147-2. The strains containing pCMsub have a reduced ability to inhibit the fungal growth, as determined by the gap between the bacterial streaks and the fungus.

5.2.4 Does PA138 have a mutation in a putative LysR-type transcriptional regulator gene?

In chapter 4, the DNA sequence flanking the Tn*5* insertion in PA138 was described. BLAST analysis suggested similarity to LysR-type transcriptional regulators (LTTRs), at the predicted translation product level. As discussed above, LTTRs are involved in regulating a number of different systems in prokaryotes, and several have been well-characterised. Further characterisation of the mutated gene in PA138 was essential in order to provide convincing evidence of a role for the gene in antifungal activity.

DNA SEQUENCING.

Further DNA sequencing was undertaken to complete the sequence of the ORF mutated in PA138, in order to improve the search for putative homologues. To obtain the desired DNA sequence, pCM138E was sequenced on both strands in its entirety. The complete sequence of the ORF that is interrupted in PA138 showed similarity to LTTRs, and was designated *finR* (fungal inhibition regulator). The predicted translation product of *finR* has greater than 30% identity with other LTTRs, which is sufficient similarity to indicate the presence of a LTTR (Schell, 1993). Figure 5.1 shows the predicted translation product of

finR aligned with a predicted LTTR from *P. aeruginosa* . The DNA sequence of *finR* is shown in appendix 4, along with a list of similar sequences and their accession numbers.

```
FinR : 1       MDKLLALKMFVQTVDSKGFSSAARQLGLATSSVTRMIDGLEAELGAVLLNRSTRQVT 57
              +D L ALK+F     + GF++AARQ+ L+ ++V++ +  LEA L   L+NR+TR ++
Match: 1   MNYPVDHLTALKVFRAVAANGGFAAAARQMNLSPAAVSKNVAELEAHLKVRLINRTTRSMS 61

FinR : 58  LSDAGSAYYLKAREILSAMADADASVTDRGDAPTGQLRISVPVAFGRRLISPHIGAFLQRY 118
           L++AG  Y  +   IL  +  ADA++T     P+G LR+S P+      ++P I AFLQRY
Match: 62  LTEAGEVYRQRLERILDDLEAADAALTSMQQGPSGLLRVSAPLTLALTCLTPAIPAFLQRY 122

FinR : 119 PDLELDMTLSDDIVDLLGERIDLSIRLGTAATMDGVVSRAIGTFRRKVVASPEYLDVRGFP 179
           P+L L++ L D   DL+ E IDL++R        G+V+R +      + A+P YL   G P
Match: 123 PELRLELLLQDGRQDLIAEGIDLALRGSDRVADSGLVARPLLVLEHVLCAAPAYLSQHGQP 183

FinR : 180 KQPTDLLQHDCMRFSYGPQQQVWTFQEDSGEVRVSVDGRFKSNNAEVLREVALAGGGVALL 240
            +P  L +H+C+RFS       WTF++D   + V + GR++ +++  +R+  LAG G++L+
Match: 184 LRPEALREHECIRFSLSGHADRWTFRKDRECIAVPIAGRYRVSSSLAVRDALLAGFGLSLI 244

FinR : 241 PDWLVSEDIESGRLTSLFESFVVNPNSASSAISALYLPNYRGSRSSRRVNAFIEFLSELL* 301
           P   V  ++  GRL  L   +     +  +AI A+    Y   + + +   F++FL+E +
Match: 245 PRLYVQAELAEGRLVELLADW----KADETAIHAV----YPSRQLAGKTRVFLDFLTETMA 297
```

Figure 5.3. Alignment of FinR with an LTTR from *P. aeruginosa*. The *P. aeruginosa* sequence is labelled "match", and the accession number is AAG05935.1. Overall, there is 34% identity, and 57% similarity between the translated products. Identical residues are shown between the two sequences, and similar residues are indicated by +. Predicted HTH motifs are shown in bold type, dashes are indicative of gaps, and *=the predicted termination codon of FinR.

5.2.5 Is *finR* expressed under antifungal bioassay conditions?

Having identified an ORF with potential importance in antifungal activity, it was of interest to determine whether the ORF was expressed under conditions relevant to fungal inhibition. Two approaches were taken to address this question.

EXPRESSION OF A *FINR*::*LACZ* FUSION.

A *finR*::*lacZ* fusion on the plasmid pFRTF833 was introduced into PA147-2 by conjugation. The transconjugant was streaked onto a buffered PDA plate in the presence of *Rhizoctonia solani*, and incubated at 22°C until inhibition zones could be seen. Bacterial cells were recovered from the plate, and β-galactosidase activity was determined as a measure of *finR* expression. The *finR*::*lacZ* fusion produced 3392.1 Miller units, compared to approximately 1000 units from the vector control, showing that FinR is expressed under bioassay conditions.

RT-PCR TO MEASURE *FINR* EXPRESSION.

As additional support to the *lacZ* fusion studies, the expression of *finR* was examined by reverse transcription PCR (RT-PCR). PA147-2 was recovered from a bioassay plate which showed clear zones of inhibition, and RNA was prepared. RT-PCR using the aforementioned RNA as a template confirmed that *finR* is transcribed under bioassay conditions (Figure 5.4).

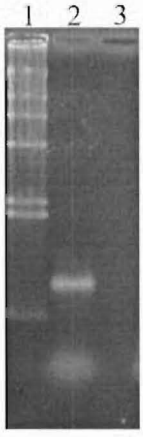


Figure 5.4. RT-PCR to examine *finR* expression under bioassay conditions. RNA was prepared from a colony showing strong fungal inhibition, and used in the RT-PCR. Lane 1 shows a lambda *Hin*dIII standard. Lane 2 is the amplified *finR*, and lane 3 shows the RT-PCR carried out after heat inactivation of the reverse transcriptase. Primers used were finexFwd and finexRev (appendix 3).

5.2.6 Deletion of *finR*.

Because the Tn*5* insertion in PA138 was near the 3′ end of *finR*, it was desirable to construct a defined *finR* null mutant. For simplicity of interpretation a deletion of *finR* was sought (see section 2.7.3). Construction of the *finR* deletion was accomplished by allele exchange between pDELKL and the PA147-2 genome. Recombination was confirmed by cloning the Km^R region from the recombinant (PA147-2*finR*Δ) and determining the DNA sequence near the ends of the cloned region (data not shown). Bioassay of PA147-2*finR*Δ indicated that deletion of *finR* resulted in the loss of antifungal activity, supporting the relevance of *finR* in this study (see Figure 5.11).

5.2.7 Computer analysis.

LTTRs have a characteristic helix-turn-helix (HTH) motif at their N-terminus (Schell, 1993). Using the program BLOCKS (http://motif.genome.ad.jp), the predicted translation product derived from the DNA sequence of *finR* was analysed for known protein motifs. This search identified a sequence at the N-terminus of FinR that strongly resembles a LysR-type HTH motif. Analysis using the program Pfam suggested that *finR* is a member of the HTH LysR family of regulators, and together with the sequence similarities and the HTH data, supports the suggestion that PA138 has an insertion in a LTTR. Thus, predictions were made based upon the knowledge of other LTTRs, and tests of these predictions were used to further analyse the insertion in PA138.

5.2.8 Predictions of the LTTR hypothesis.

Several predictions can be made based upon the hypothesis that *finR* is a LTTR. These are listed here, and details of their rationale and testing are provided below.

A divergently transcribed open reading frame (ORF) will be present downstream of *finR*.

A T-N_{11}-A motif with interrupted dyad symmetry will exist between the coding sequences of *finR* and the divergent ORF.

The downstream ORF will be regulated by FinR.

The downstream ORF will be involved in antifungal activity.

IS THERE A DIVERGENT ORF?

A common feature of LTTRs is the presence of an adjacent ORF expressed from a divergent promoter (Schell, 1993). Thus, it was reasonable to examine the DNA region immediately upstream of *finR* for the presence of an ORF. This would also reveal the sequence of the promoter region of *finR*, providing further information that could contribute to its classification as a LTTR (see below). The DNA sequence upstream of *finR* was subsequently obtained. Computer analysis suggested that there was an ORF of 942bp, and BLAST queries of public databases revealed that the predicted translation product had similarity to a family of reductase enzymes. The DNA sequence of this ORF (designated *finA*) is shown with that of *finR* in appendix 4, and the arrangement of the genes is illustrated in Figure 5.5.

Figure 5.5. Diagrammatic representation of the organisation of *finR* and *finA*. The open reading frames are divergent, and separated by an 182 bp intergenic spacer region.

IS THERE A T-N_{11}-A MOTIF BETWEEN *FINR* AND *FINA*?

The intergenic region between a LTTR and its target gene should have the promoters for both genes, characterised by the presence of –10 and –35 consensus sequences (Schell, 1993). In addition, LTTRs recognise a sequence of T-N_{11}-A, with interrupted dyad symmetry, often with symmetrical guanines that are important for binding via HTH motifs (Jordan & Pabo, 1988). Examination of the DNA sequence between *finR* and *finA* reveals the presence of a putative LTTR recognition site (Figure 5.6).

5′-TGCGCATTGCGCA-3′

3′-ACGCGTAACGCGT-5′

Figure 5.6. Putative *finR* recognition sequence (T-N_{11}-A motif) found between *finR* and *finA*. Dyad symmetry is underlined. Note the presence of symmetrical guanines.

IS *FINA* REGULATED BY FINR?

The arrangement of ORFs at the locus mutated in PA138 is consistent with a LTTR model in which FinR would regulate expression of *finA*. To test the hypothesis that *finA* is controlled by FinR, a number of *finA*::*lacZ* fusions were constructed, and β-galactosidase expression was used as a marker for FinA.

Expression of FinA from pFATF833.

The plasmid pFATF833 has a *finA*::*lacZ* fusion, and includes both the intergenic region between *finR* and *finA*, and the first 45bp of *finR* upstream of *finA*. The rest of the *finR* gene was replaced by an omega cassette, which provides transcriptional and translational stop signals. Although the precise location of the *finA* promoter was not known, it was

expected that any FinA expression would be a result of activity of the *finA* promoter, since the omega cassette should prevent expression from other promoters on the plasmid. To investigate the possible influence of *finR* on *finA*, pFATF833 was introduced into PA147-2 (*finR*$^+$) and PA138(*finR138*) and β-galactosidase assays were carried out for both strains after growth in buffered potato dextrose broth (PDB) at 25°C. The medium and incubation temperature were chosen because of the relevance to the synthesis of antifungal compounds by PA147-2. Preliminary investigations into the antifungal compound have revealed that PA147-2 produces inhibitory compounds in buffered PDB culture grown at 25°C. Overnight growth (18 hours) resulted in very similar β-galactosidase levels from the two strains (Figure 5.7), suggesting that FinR had little influence on *finA*.

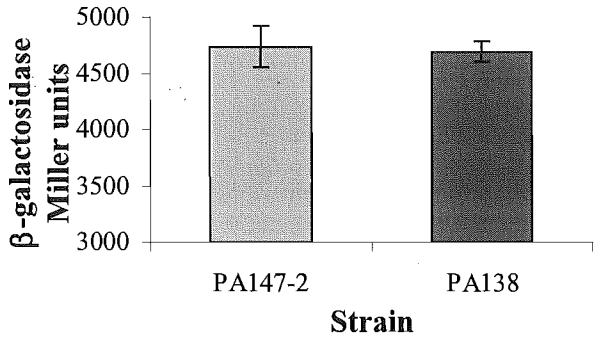


Figure 5.7. β-galactosidase expression from PA147-2(pFATF833) and PA138(pFATF833) grown overnight in buffered PDB. Data shown are the averages from three separate experiments. Standard errors are shown

Possible reasons for the lack of difference between FinA expression by PA147-2 and PA138 include the fact that the assays were carried out in broth culture, whereas fungal inhibition assays are conducted on agar plates, and the possibility that the presence of the fungus stimulates antifungal activity. To examine the effects of solid versus liquid growth, and the effect of time on gene expression, two further experiments were carried out. Firstly, PA147-2(pFATF833) and PA138(pFATF833) were inoculated onto buffered PDA plates, with and without fungi present. When the inhibition zones produced by PA147-2 were clearly visible, bacteria were removed from the plates, and β-galactosidase assays were performed. These results (Figure 5.8) supported the initial findings from broth culture that FinR appeared to have little effect on *finA*::*lacZ* expressed from pFATF833, and also suggested that the presence of the fungus has no role in the induction of FinA expression.

An alternative approach would be to examine induction of *lacZ* using a protein gel stained for β-galactosidase. Although the data show that FinA expression is slightly higher in the presence of fungus, the difference is not great.

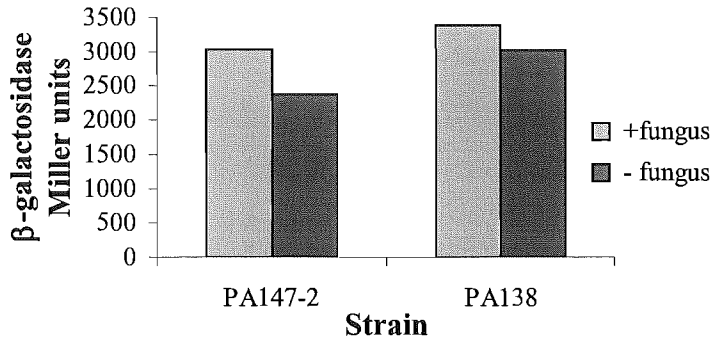


Figure 5.8. β-galactosidase expression from PA147-2(pFATF833) and PA138(pFATF833) from antifungal bioassays. The strains were grown on buffered PDA in the presence or absence of *G. graminis* var. *tritici*. These data are the result of a single experiment.

Secondly, a time course experiment was conducted in buffered PDB. Duplicate cultures of PA147-2(pFATF833) and PA138(pFATF833) were grown for 400 hours, and β-galactosidase activity was measured periodically. These data (Figure 5.9) demonstrate that FinA expression is not greatly different between the two strains over the initial 150 hours, although the strains do differ after this time point, with FinA expression from PA138 increasing approximately two-fold. The difference is unlikely to be important in antifungal activity since the inhibition assays are generally complete before 150 hours of incubation.

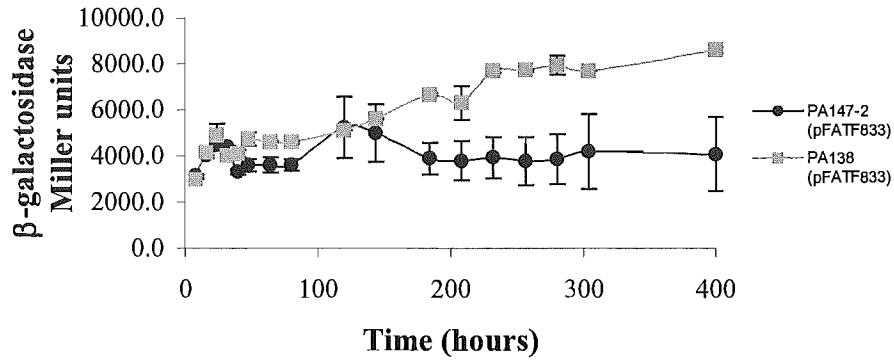


Figure 5.9. Time course experiment measuring β-galactosidase from PA147-2(pFATF833) and PA138(pFATF833). Data shown are from two independent cultures. Standard errors are shown.

It appears from the data above that FinA may not be regulated by FinR, at least under the conditions tested. However, a report on the *ilvYC* operon of *E. coli* suggested that the characteristic organisation of the divergent promoters in LTTR systems has evolutionary significance, and is important for regulation of the gene adjacent to the regulator gene (Rhee et al., 1999). Since the construct pFATF833 has only 45bp of *finR* present, the expression of FinA from the plasmid might not reflect what actually occurs when *finA* is encoded divergent from *finR* and *finR138*. To test the possibility that the *finR* or *finR138* influence on *finA* was dependent upon the proximity of the *finR* alleles, pFATF1000 and pFATF2000 were constructed (see section2.7.3, pp 52-53). Both of these new constructs had a *finA*::*lacZ* fusion, but differed in the region upstream of *finA*. pFATF1000 had a wildtype *finR* gene in an identical arrangement to that found in PA147-2, while pFATF2000 had the *finR138* allele in place of *finR*. Expression of FinA from these plasmids would address the significance of the genetic arrangement of LTTR loci, and extend the examination of the possible effect of *finR138* on *finA*.

FinA expression from pFATF1000 and pFATF2000.

pFATF1000 and pFATF2000 were constructed to test the hypothesis that the influence of FinR on FinA was at least partly due to the genetic arrangement of *finR* and *finA*. The two plasmids were separately introduced to PA147-2 by conjugation, and β-galactosidase levels were measured after overnight growth in buffered PDB. Contrary to the findings with

pFATF833, in which the presence of *finR138 in trans* had no significant impact on FinA expression, there was a considerable difference in FinA expression between pFATF1000 and pFATF2000 (Figure 5.10). It is likely that this difference can be attributed to the fact that pFATF1000 has the wildtype *finR* allele present, while pFATF2000 has the *finR138* allele.

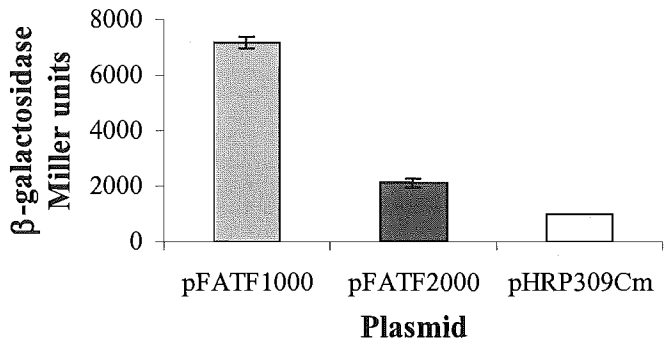


Figure 5.10. β-galactosidase expression from PA147-2 containing either pFATF1000 (*finR*$^+$), pFATF2000 (*finR*$^-$), or pHRP309Cm (vector control). β-galactosidase levels are indicative of the level of FinA expression from each plasmid. These data are the average of four independent experiments for pFATF1000 and pFATF2000, and three experiments for pHRP309Cm. Standard errors are shown.

With an intact *finR* gene *in cis*, β-galactosidase expression is approximately 7000 Miller units, compared to 2000 units when the *finR138* allele is present, and 4000 units from pFATF833, in which neither *finR* allele is present *in cis*. Table 5.2 shows the levels of β-galactosidase expression when the background level from the plasmid vector is removed. Further support for the *cis* requirement of *finR* comes from *finA* expression experiments in which *finR* was present *in trans* on a plasmid (pFin3M). These experiments demonstrate that addition of *finR in trans* does not increase β-galactosidase levels from pFATF1000 or pFATF2000 (Table 5.2).

Table 5.2. Expression of β-galactosidase from *finA*::*lacZ* fusions.

Plasmid	*finR* allele (*cis* and *trans*)	FinA expression[a]	Relative expression level[b]
pFATF833	None	3735.1	3.2x
pFATF1000	*finR* (*cis*)	6166.8	5.3x
pFATF2000	*finR138* (*cis*)	1167.0	1x
pFATF1000 and pFin3M	*finR* (*cis*) *finR* (*trans*)	6935.6	5.9x
pFATF2000 and pFin3M	*finR138* (*cis*) *finR* (*trans*)	905.3	.78x

[a] Expression of FinA is expressed in Miller units of β-galactosidase expressed from the relevant fusion construct. These data have had the background level of β-galactosidase expression (from the vector) subtracted.
[b] Relative to expression from pFATF2000, which is arbitrarily assigned 1x.

IS FINA INVOLVED IN ANTIFUNGAL ACTIVITY?

The final prediction of the LTTR hypothesis was that the adjacent ORF (*finA*) would be involved in antifungal activity. This was based upon the putative identification of *finR* as a LTTR, which suggested it would regulate an adjacent ORF. Since the *finR* mutation rendered PA138 antifungal deficient and the likely function of *finR* was as a regulator of the adjacent gene, it was reasoned that the adjacent gene was probably involved in fungal inhibition.

To investigate the possibility that *finA* is involved in fungal inhibition, a *finA* mutant was constructed. The mutation was made by introducing a Gm^R gene into the *finA* coding sequence of the clone pRED2L. Specifically, the Gm^R gene interrupts *finA* at base 606, thus creating the *finA606* allele. The resulting clone, called pRED2Lgm, was introduced into PA147-2 by conjugation, and *finA* was replaced by *finA606* via allele-exchange. An *in vitro* bioassay revealed that the resulting PA147-2*finA606* strain was reduced in its ability to inhibit fungal growth (Figure 5.11). Thus, it appears that *finA* is necessary for complete inhibition of fungal growth, but is not in itself sufficient for antifungal activity. The sequence analysis of FinA is not revealing in terms of possible roles for FinA in antifungal activity, since no similar proteins are known to contribute to *in vitro* antibiosis. The best studied member of the broad flavoprotein reductase family is old yellow enzyme (OYE)

found in several yeast species. However, the physiological role of OYE is unclear (Kohli & Massey, 1998). Similar proteins are known to be capable of catalysing the denitration of nitrate esters (Blehert et al., 1997; French et al., 1996) and the reduction of α, β-unsaturated aldehydes and ketones (Kohli & Massey, 1998). Some of these flavoprotein reductases have roles in degrading xenobiotic compounds such as TNT and nitroglycerine (Blehert et al., 1999; French et al., 1998).

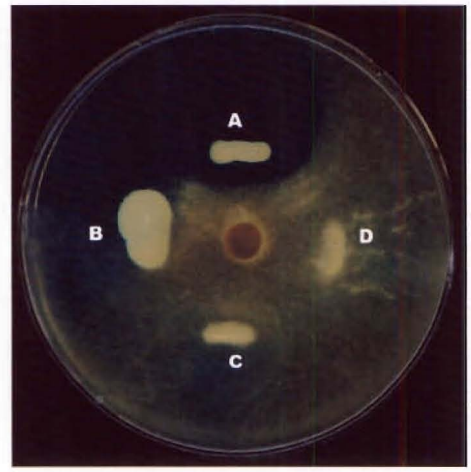


Figure 5.11. Bioassay results of *finA*- and *finR*-defective isolates. Samples: wildtype PA147-2 (A), PA147-2*finA606* (B), PA147-2*finR*Δ (C), and PA138 (D), assayed against *R. solani*. While PA147-2 inhibits the fungal growth, both PA138 and PA147-2*finR*Δ are unable to inhibit the fungus, and become overgrown by it. PA147-2*finA606* has reduced inhibitory activity. The fungus can grow to the colony edge, but cannot grow over the colony.

5.3 Analysis of a putative two-component global regulator.

5.3.1 Complementation of the mutation in PA109.

In a previous study, the mutation in PA109 was complemented by allele-exchange, as a high rate of homologous recombination hampered *trans* complementation experiments (Carruthers et al., 1994). In chapter 3 of the present study, the construction of *recA* strains of PA147-2 and its mutant derivatives was described. Since the failure of *trans* complementation was believed to be due to recombination, it was decided to attempt complementation in PA109*recA*gm. The Tn*5* insertion in PA109 lies in a 16kb *Eco*RI fragment. The plasmid pAF16M is a clone of that 16kb *Eco*RI region, and the plasmids pAF2M, pAF6M, pAF7M, pAF8M, and pAF9M are all subclones of pAF16M (Figure 5.12b). These plasmids were all transferred to PA109*recA*gm by conjugation, and the

resulting transconjugants were assessed for their ability to inhibit the *in vitro* growth of *G. graminis* var. *tritici*. The results are shown in Figure 5.12. It appears from these results that the gene interrupted in PA109 is encoded by pAF8M, and that the gene spans the *Bam*HI site. This is based upon the observation that neither pAF2M nor pAF6M could complement the mutation, indicating neither clone contained the complete gene that was disrupted in PA109. However, pAF8M, which covers both pAF2M and pAF6M was able to complement the mutation in PA109, allowing the conclusion that both pAF2M and pAF6M encoded part but not all of the gene of interest. It is also noteworthy that complementation by pAF8M appears to result in a larger zone of inhibition than that produced by PA147-2, suggesting the possibility that multiple copies of the regulator gene can up-regulate expression of antifungal compounds.

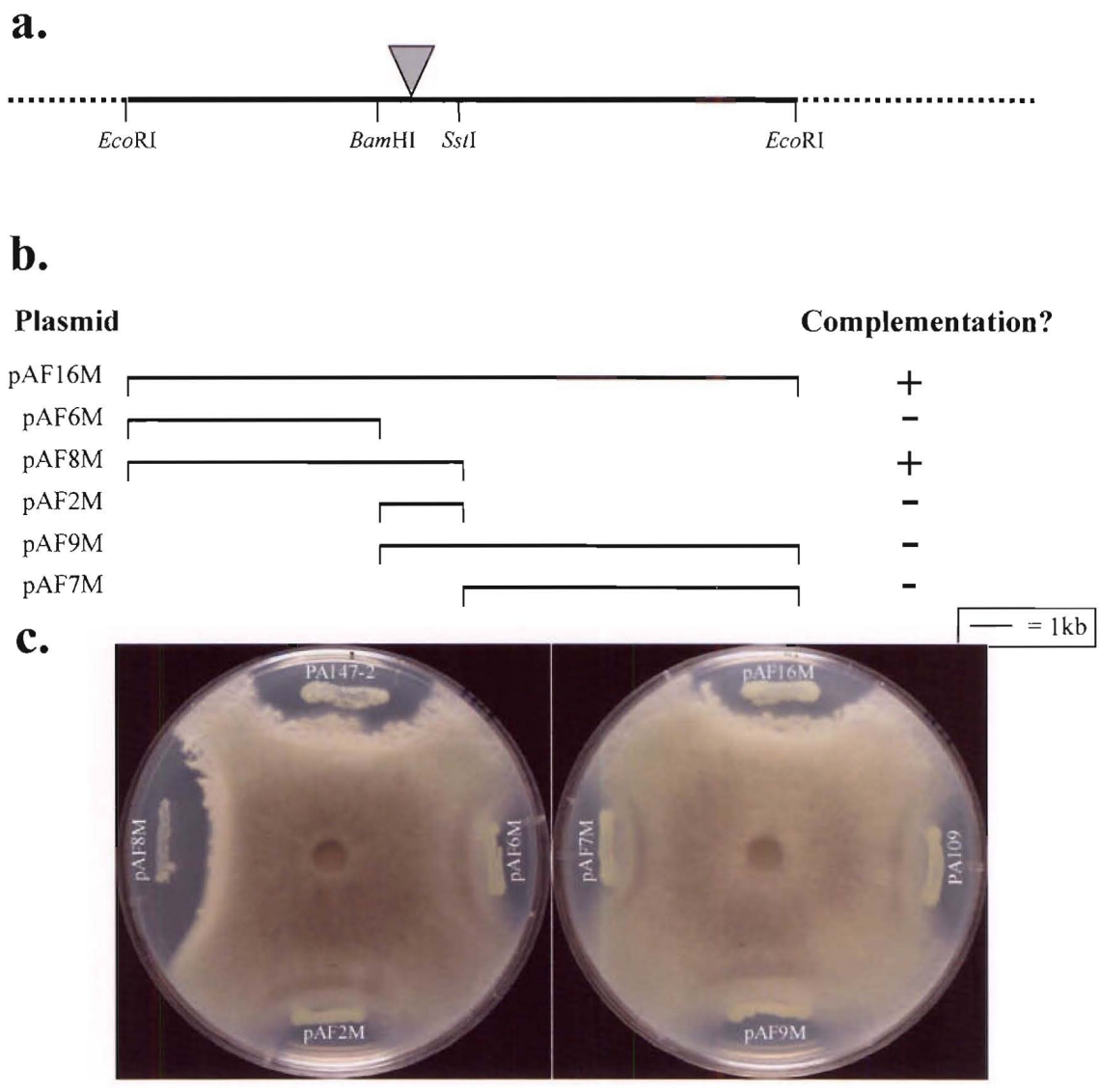


Figure 5.12. Complementation of the mutation in PA109. **A**. Diagrammatic representation of a 16kb *Eco*RI region from PA147-2, that is interrupted by Tn*5* in PA109. The transposon is indicated by a triangle. **B**. Illustration of the various subclones of the 16kb region that were used in complementation. Their ability to complement the mutation in PA109 is indicated. **C**. Bioassays of PA109 complementation experiments. PA147-2 and PA109 were used as controls. Plasmid names on the figure represent the plasmid in a PA109 background. The fungus used was *G. graminis* var. *tritici*.

5.3.2 DNA sequence of the mutated gene in PA109.

The DNA sequence presented in chapter 4 indicated that PA109 had a Tn*5* insertion in a gene probably encoding a two-component regulator. There are a number of different classes of two-component regulators, so in order to verify the identification and aid in classification, the putative two-component regulator gene interrupted in PA109 was sequenced. Using the complementation data it was determined that the gene of interest was

encoded by pAF8M, and that part of the gene was found within pAF2M. This was in agreement with the location of the Tn*5* insertion previously determined (Carruthers et al., 1994). To obtain the DNA sequence, the cloned regions in pAF2M and pAF8M were cloned in pBLUESCRIPT KS-, and DNA sequence was determined. The complete DNA sequence of the probable two-component regulator gene (designated *finT*) is shown in appendix 4. The highest scoring sequence in a Blastx search is shown aligned with FinT in Figure 5.13.

```
FinT : 1     MTFRRRWDINTRTQLITLGPALLLTLLLISFFTFVRIQDLRQELDHTGQFIANQLAPATE 180
               M+ R  WDI TR    I+LGPALLLTLLL ++FT+ R+QDLRQEL HTGQ IA+QLAPA E
Match: 1     MSQRHGWDIRTRMLAISLGPALLLTLLLTAYFTYSRLQDLRQELTHTGQLIADQLAPAAE 60

FinT : 181   YGVISGNNDVLESLLRATLATPHVRSWRFRTCCRKYPGVCRATVGEARSLAVGESLPGAD 360
               YGVI+GN  VL+ LL+ATL TPHVR     R                G  ++ A  +
Match: 61    YGVIAGNTPVLQKLLQATLDTPHVRFIEVRDRNDNILVYVEQLSGALQNAAPIDIFHSTI 120

FinT : 361   SLQHIQLGNDFFQDSTAEPKAPRA-DYLGRVIVGMSNDAFSQRQQEILFKAGILALSPCC 537
                 Q I L +D    D   +E         DYLGRV+VGMSNDAFSQRQQEIL KA +LA
Match: 121   QRQRIALASDPLLDGASEGDGQSGEDYLGRVVVGMSNDAFSQRQQEILLKAALLAAFALI 180

FinT : 538   LPSAGPAPGAS-LSQPISAMGNAVKAIQQGDYQTPLPIVDDSELGT-CRHINNLADALTR 711
               L         A  LS PIS MG AV+AIQ GDY+T LPI+DD E+G    RHINNLA  L R
Match: 181   LTFLVARRLAQRLSAPISTMGQAVEAIQSGDYKTSLPILDDGEIGDLARHINNLASGLDR 240

                                                  H
FinT : 712   PVVNSTRPWPGL-SRPAKKPSGNNAKSDFLAM MSHELRTPM NGVLGMLQLLETTDMTEEQ 888
                    +     L S    +     N AKSDFLAM MSHELRTPM NGVLGMLQLLETT+ T EQ
Match: 241   ASREQEQAIGQLISAREEAEQANRAKSDFLAM MSHELRTPM NGVLGMLQLLETTEQTREQ 300

FinT : 889   TEYAALASEVHRTPAEGDQRHPRLLAHRAGPLELEHIPFDLVELIGSCAQAFQHAAQQRG 1068
               EY ALA+E       +                G LELE IPF+L+EL+    A  FQH+AQQRG
Match: 301   AEYTALATESTEHLLKVINDILDFSRIERGALELECIPFNLLELVQGSALVFQHSAQQRG 360

                                                  N
FinT : 1069  LALEVPIPQGLGSLQVQGDPTR IRQILVNLIGNA LKFTEQGTVTVEPHWQTLDHELLWFT 1248
             LALE+ I  GL +++V GDPTR IRQILVNL+GNA LKFTE+G + +    WQ LDH++LW T
Match: 361   LALELQIQAGLENIEVCGDPTR IRQILVNLLGNA LKFTEEGAIHLSLEWQALDHDVLWLT 420

                    G1                F                          G2
FinT : 1249  CT VRDSGIGIS AERLEL MFDAF QQADSSISRRYG GTGLGL PIARTLAERMGGTLRAQSEE 1428
             C  V DSGIGIS  ERLE  MFDAF QQADSSISRRYG GTGLGL  IARTLAERMGGTL+A+S+E
Match: 421   CA VHDSGIGIS PERLEH MFDAF QQADSSISRRYG GTGLGL AIARTLAERMGGTLQAESKE 480

                                                          Asp-13
FinT : 1429  GHGSVFTLEIPLAIYQQSLPVLAPNTEGNGR-AGEGRNVLLVE D NPVNRTVVEACLRSLG 1605
             G GS FTLEIPL  +QQS P        G+       G+ +LLVE D NPVN+TV+EA LRSLG
Match: 481   GSGSTFTLEIPLP-FQQS-PAHRQQAAGDAAPVAAGQEILLVE D NPVNQTVIEAMLRSLG 538

                                                  Asp-57
FinT : 1606  FEVSIATDGAEAIRSAESLIFTAILM D CRLPGIDGYEATRQIRQLPGCAELPIIA--SRP 1779
             + V++  DG +A+RSAE   + AILM D CRLP +DGY ATR+IR        +PIIA  +
Match: 539   YRVTLVADGIQAVRSAERQRYDAILM D CRLPVLDGYSATREIRAQENGRRVPIIALTANA 598

                                        Lys-109
FinT : 1780  MLAGRSGSLPGSWNERLPA K PFKRTDLQQILQRWVQ*1884
             +   R   L    N+ L A K PFKR +LQ+ILQRW+
Match: 599   LQGDRENCLQAGMNDYL-A K PFKRAELQRILQRWI 632
```

Figure 5.13. Alignment of FinT with a probable two-component regulator from *P. aeruginosa*. These sequences are 54% identical, and 63% similar. The accession number for the *P. aeruginosa* protein is AAG05000.1. The sequence shown starts at the predicted initiation codon, and finishes at the predicted stop codon (*). Conserved features of two-component regulators are shown in boxes, labelled in the alignment. The Asp-13, Asp-57, and Lys-109 positions are those suggested by Carruthers (1994). The H, N, G1, F, and G2 boxes were found manually, and are based upon consensus sequences described by Parkinson and Kofoid (1992).

The results of database searches using the Blastx program support the suggestion that *finT* encodes a global regulator protein, and indicate that this protein is probably of the sensor/response regulator hybrid type. The possibility that the H, N, G1, F, and G2 sequences (features of the transmitter portion of the sensor proteins), and the conserved Asp-13, Asp-57, and Lys-109 of response regulators are found on the same protein lends additional support to the hybrid organisation. Block H contains the histidine residue that is autophosphorylated, and serves to transmit the phosphorylation signal to Asp-57 in the cognate response regulator (Parkinson & Kofoid, 1992).

5.3.3 Maxicell protein analysis.

In the previous study (Carruthers, 1994), it was suggested that the two-component regulator regulates a number of genes found in close physical proximity to it. Specifically, it was thought that these genes were located within the same 16kb *Eco*RI fragment that encodes *finT*. One approach to address this hypothesis was the expression of proteins using *E. coli* maxicells. *E. coli* CSR603 is highly sensitive to UV irradiation due to *recA1*, *uvrA6*, and *phr-1* mutations (Sancar et al., 1979). Thus, UV exposure leads to chromosome degradation. Proteins expressed from medium copy number plasmids can be studied by transforming CSR603 with the plasmid DNA, then subjecting the transformed strain to UV irradiation. While chromosome degradation will be considerable, it is likely that at least some plasmid molecules will survive because of their copy number and relatively small size (compared to the *E. coli* chromosome). Once the chromosome is destroyed, radioactively labelled amino acids can be added to the culture, and these will be incorporated into proteins synthesised *de novo*. Since there is little or no chromosomal DNA, most proteins will be synthesised from plasmid-based genes. SDS-PAGE followed by autoradiography allows plasmid-encoded proteins to be visualised with minimal contamination by proteins encoded on the chromosome.

E. coli CSR603 was transformed with the plasmids pWT109, pFC109, pBR322, and pACYC184, and the proteins encoded by these plasmids were examined. pWT109 is a pACYC184-based clone of the wildtype 16kb *Eco*RI region (Figure 5.12a), and pFC109 is a clone of the 16kb::Tn*5* in pBR322. Thus, if *finT* is important for the expression of

proteins encoded by the 16kb region, there should be a clear difference between the protein profiles of pWT109 and pFC109, and the fact the clones were constructed using different vectors would be insufficient to explain these differences. From the results (Figure 5.14) it is clear that there is no significant difference in proteins expressed by the two plasmids. There are at least two possible explanations for the maxicell results. The first possibility is that FinT does not positively regulate expression of genes encoded within the 16kb region under investigation. The second scenario is that *finT* cannot be expressed in *E. coli*, or is not expressed in sufficient quantity, so even if there were genes under FinT control, they would not be expressed due to a lack of FinT protein. The second suggestion is supported by examination of the maxicell protein gel and autoradiograph. Based upon DNA sequence analysis, FinT is predicted to be 68.7kDa, but there is no highly expressed protein of this size evident on the autoradiograph, suggesting FinT is not well expressed in *E. coli*. These two possible explanations are not mutually exclusive, but can both be tested.

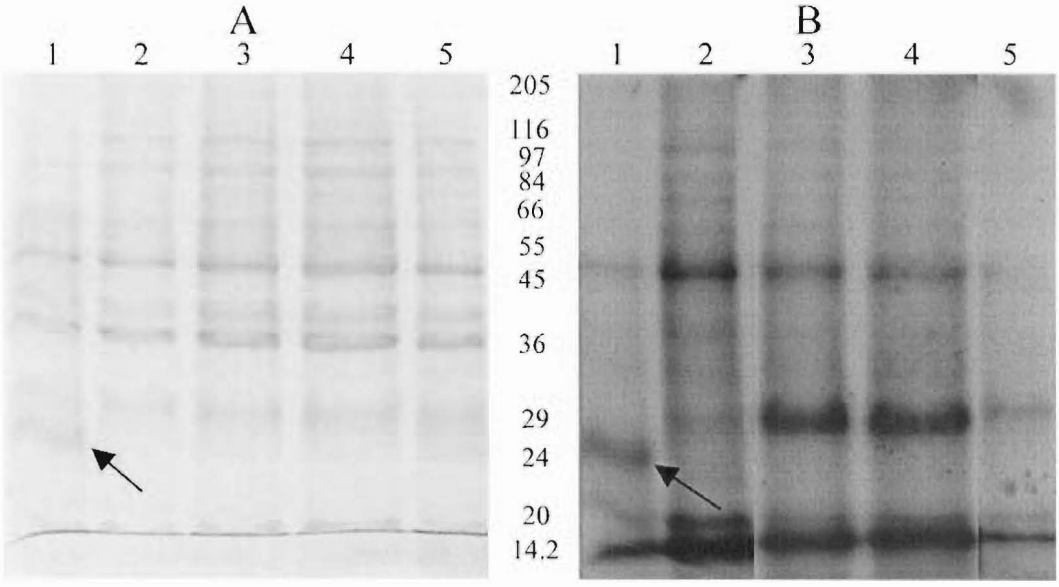


Figure 5.14. Expression of proteins encoded by clones of the 16kb *finT* region using *E. coli* maxicells. **A.** 12% SDS-PAGE gel stained with coomassie blue. **B.** Autoradiograph of the gel shown in A. In both panels, all lanes show expression of proteins from plasmid-encoded genes. The plasmids were, lane1, pACYC184; lane 2, pWT109; lane 3, pFC109; lane 4, pFC1; lane 5, pBR322. No differences can be observed between proteins from the wildtype 16kb (pWT109), or the two clones bearing Tn*5* insertions in the 16kb region (pFC109 and pFC1), indicating that no FinT regulated genes were expressed in this experiment. The arrow indicates the Cm^R protein from pACYC184. Note that it is absent in lane 2, due to the fact that the 16kb region is cloned into the *Eco*RI site, which is in the Cm^R gene. Sizes in kDa are shown between the panels.

5.3.4 Saturation mutagenesis.

Saturation mutagenesis was used to further investigate the possibility that there are antifungal genes clustered in the 16kb *Eco*RI region. The mutagenesis approach was chosen to complement the maxicell analysis, which showed no evidence of FinT-regulated gene expression. While not testing the regulation of genes by FinT, the use of mutagenesis would directly test the theory that antifungal genes are found in the 16kb region by creating a range of mutants.

The clone pAF9 was subjected to insertional mutagenesis using miniTn*10*km (Kleckner et al., 1991). Seven mutated plasmids were recovered and their insertion point mapped (Figure 5.15a). Each mutant was separately introduced into PA147-2 by conjugation. The transconjugants were grown in the absence of selection to encourage recombination between the particular plasmid and genome. Recombinant strains were enriched by selecting Km^R colonies, and selected by screening for Tc^S colonies. Recombinants were generated with each of the seven mutated pAF9 plasmids, and recombination of five of these was confirmed by Southern analysis (Figure 5.15b). The recombinant strains were designated PA147-9.X, where X represents the particular transposon insertion point (as shown in Figure 5.15a). *In vitro* bioassays were used to assess the impact of the various insertions on antifungal activity. These assays showed that all recombinants were still able to inhibit the growth of *Gaeumannomyces graminis* var. *tritici*, indicating that the insertions had not interrupted sequences important for antifungal activity.

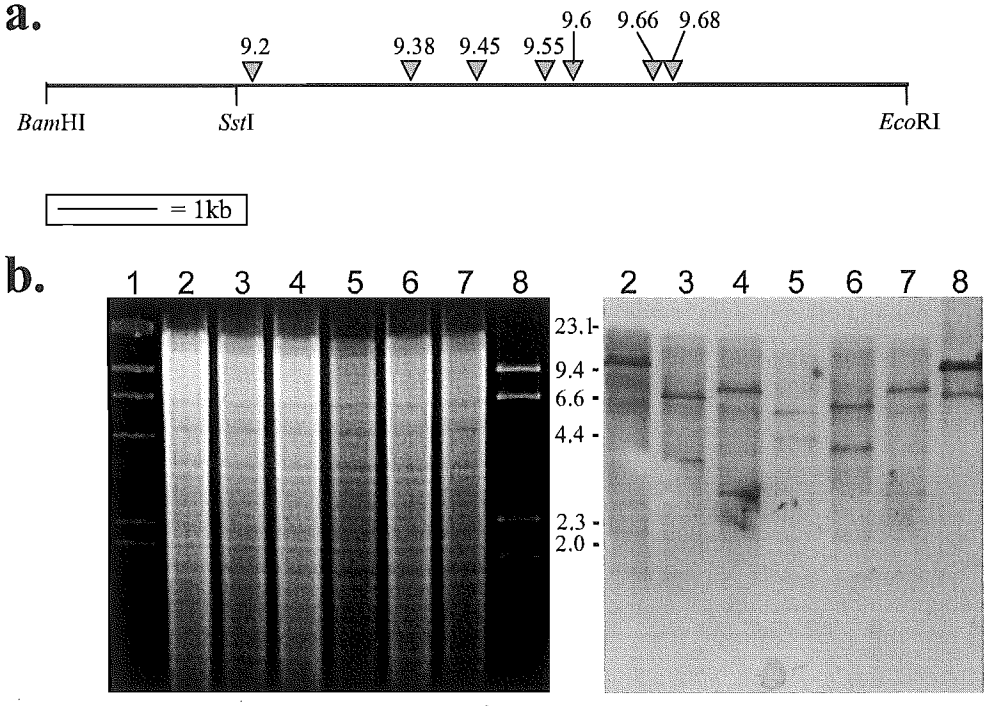


Figure 5.15. Mutagenesis of pAF9 and introduction of mutations into the PA147-2 chromosome. **a.** Diagrammatic representation of the miniTn*10* insertions in pAF9. Transposons are represented as triangles. **b.** Southern analysis of five recombinant strains. Each has a miniTn*10* in the 9kb *Bam*HI/*Eco*RI region cloned in pAF9. Lane 1 is a lambda *Hin*dIII standard. Lane 2 is a PA147-2 control. Lanes 3-7 are recombinants PA147-9.6, PA147-9.68, PA147-9.45, PA147-9.38, and PA147-9.66 respectively. All genomic DNA was digested with *Bam*HI and *Eco*RI. Lane 8 shows *Bam*HI/*Eco*RI digested pAF9. The membrane was probed with the 9kb region from pAF9. Hybridisation to two bands in the recombinant lanes is expected because the miniTn*10* has two *Bam*HI sites, thus splitting the 9kb into two pieces. The absence of the 9kb in the recombinants is strong evidence for successful allele exchange mutagenesis.

5.4 Protein profiles of PA138 and PA109.

The possibility that PA138 and PA109 have mutations in global regulator genes suggested that it might be possible to observe the regulatory effects of the mutations by SDS-PAGE. After 24 hours of growth in media that supports antifungal compound synthesis, total soluble protein from PA147-2, PA138, and PA109 was examined using SDS-PAGE, and proteins synthesised *de novo* at the end of 24 hours were examined by labelling with ^{35}S-labelled methionine and cysteine, SDS-PAGE, and autoradiography. Total protein profiles (Figure 5.16a) revealed that there was at least one protein present in PA147-2 that was absent in both mutants. Examination of the autoradiograph (Figure 5.16b) indicates that in addition to the difference noted on coomassie-stained SDS-PAGE gels, there were at least two more proteins present in PA147-2 and absent in both mutants. Furthermore, it is interesting to note that it seems the mutants make some proteins that PA147-2 does not

make. This is interesting because it adds weight to the suggestion that *finR* and *finT* encode regulators, and also because it suggests that FinR and FinT may have common regulatory targets.

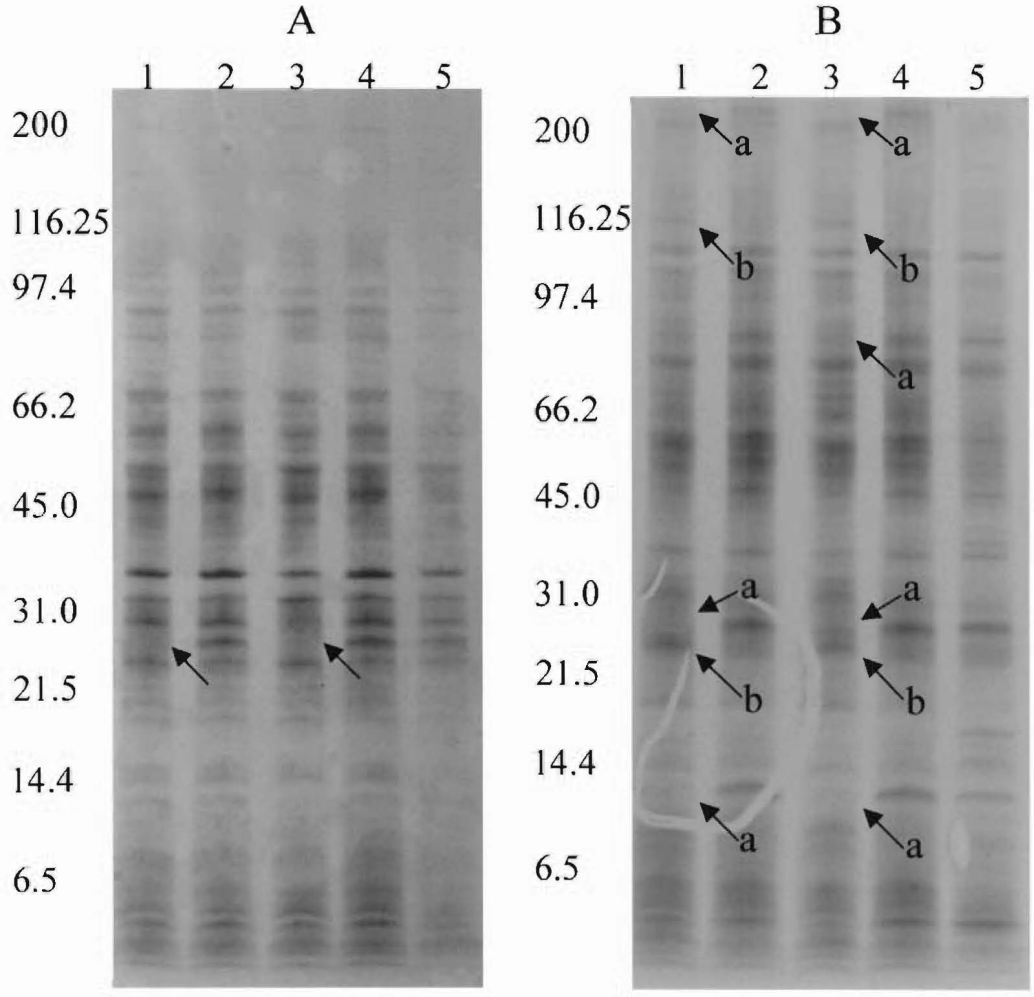


Figure 5.16. Comparison of proteins expressed by PA147-2 and mutants PA109, PA138, and PAH26. **A.** Coomassie blue stained SDS-PAGE of total protein from PA147-2, PA109, and PA138. The arrows show the protein missing in PA109 and PA138. This protein was subjected to N-terminal sequencing. **B.** Autoradiograph of the gel shown in **B**. Proteins were labelled with ^{35}S-cysteine and methionine for one hour after 24 hours growth. Arrows labelled "a" indicate places where proteins are missing in PA109 and PA138. Arrows labelled "b" indicate proteins expressed by PA109 and PA138 that are not seen in PA147-2 extracts. In both panels, lane 1, PA109; lane 2, PA147-2; lane 3, PA138; lane 4, PA147-2; lane 5, PAH26.

In an effort to identify one of the regulatory targets, N-terminal sequence was obtained for the protein whose differential expression was easily observed in coomassie blue stained SDS-PAGE gels (arrowed in Figure 5.16a). Eight residues from the N-terminus were obtained, and the ScanProsite tool on the ExPASy www server (http://expasy.proteome.org.au/tools/scnpsit2.html) was used to search the Swissprot database for similar proteins. No matches were found for the sequence (Figure 5.17), suggesting that one target for FinR and FinT regulation encodes a previously uncharacterised protein.

[Ser/Ala] – Val – Leu – Thr – Xxx – Thr – [Leu/Gln] - Lys

Figure 5.17. Deduced N-terminal amino acid sequence of the protein indicated in Figure 5.16a. Xxx indicates an undefined residue, and residues in square brackets indicate ambiguity.

5.5 Discussion.

The results presented in this chapter represent the preliminary characterisation of two putative global regulators of antifungal activity in *P. aureofaciens* PA147-2. Although global regulation of secondary metabolism has been extensively studied in fluorescent pseudomonads, the emerging complexity of regulatory circuits in other situations indicates that the picture is far from complete (Schell, 2000). Thus, the studies presented here add to the growing picture of secondary metabolite regulation.

5.5.1 Analysis of a two-component regulator.

The DNA sequence of a putative two-component regulator gene (designated *finT*) was completed to examine the possibility that it was a homologue of *gacA* or *gacS*, which encode a well characterised two-component system in pathogenic and biocontrol pseudomonads (Kitten et al., 1998; Laville et al., 1992; Rahme et al., 1995). The results of database searching using the Blastx algorithm show that FinT has some similarity to GacS (accession number AAG13658.1). The proteins are 27% identical, but FinT also has 27% identity with RtpA from *P. tolaasii* (accession number BAA34717.1). Furthermore, FinT

appears to be more similar to two-component sensor/regulator hybrids, suggesting that it is not a homologue of either GacA or GacS. The predicted protein sequence of FinT contains sequences that are consistent with features of both sensors and response regulators, which adds additional support to the classification of FinT as a hybrid sensor/regulator.

Expression of *finT* in a *finT* isolate of PA147-2 (shown in Figure 5.12) suggests that multiple copies of *finT* can lead to increased antifungal activity. This indicates that regulation by FinT might be dose dependent. If PA147-2 were to be developed further as a biocontrol strain, it would be useful to examine the possibility of enhancing the strain by integrating one or more additional copies of *finT* into the genome, with a view to over-expressing antifungal metabolites.

FINT DOES NOT ACT LOCALLY.

In a previous study it was suggested that FinT regulated genes are found in close proximity to the *finT* gene (Carruthers, 1994). This suggestion was based upon mutagenesis of a 16kb *Eco*RI fragment from PA147-2, which contained the sequence for *finT*. In this thesis, two approaches were taken to further examine FinT gene regulation. Saturation mutagenesis of a 9kb region adjacent to *finT* was carried out to identify genes important in antifungal activity, and maxicell protein studies were performed to compare proteins expressed from clones of the 16kb *Eco*RI region containing either wildtype or mutated *finT*. Mutagenesis failed to identify sequences that were important for fungal inhibition, while the maxicell protein experiments did not indicate any major differences between proteins expressed in the presence of wildtype and mutated *finT*. Taken together, these results indicate that, contrary to the earlier study, FinT probably does not regulate genes found in close proximity to the *finT* coding sequence. The apparent discrepancy between the mutagenesis results reported here, and those reported previously (Carruthers, 1994) could possibly be explained by an independent loss of antifungal activity during the allele-exchanges carried out by Carruthers. In chapter 8 of this volume, experiments indicate that while in culture PA147-2 can lose the capacity to inhibit fungal growth. It is therefore possible that the mutants examined by Carruthers (1994) were not generated by allele-exchange as supposed, but were the result of concomitant, spontaneous loss of antifungal activity. Given that five of seven insertions within the 16kb region that were introduced to the

PA147-2 genome were reported to be antifungal-deficient, it seems improbable that each was the result of spontaneous mutation, but this explanation cannot be ruled out. An additional approach to address the question would be to re-examine the mutations created by Carruthers (1994). No Southern hybridisation data is presented in that study to support the introduction of the miniTn*10* insertions into the genome by allele-exchange. Thus, the possibility exists that the putative recombinants were the result of illegitimate recombination, which would generate the Km^R Tc^S phenotype, but not the introduction of the miniTn*10* into the desired chromosomal location. Such events have been observed to hamper allele-exchange in PA147-2 throughout the course of the present study (data not shown).

The results from the maxicell protein analysis support a conclusion that FinT does not regulate the expression of any genes within the 16kb region. However, the possibility that the PA147-2 genes could not be properly expressed in *E. coli* was not excluded. If *finT* could not be efficiently expressed due to poor promoter recognition or different codon preferences in *E. coli*, it would be difficult to observe gene regulation by FinT. The observed result from the maxicell experiment is consistent with the conclusion that FinT does not regulate a proximal gene, but is also consistent with the alternative suggestion that *finT* is not properly expressed in *E. coli*. Although there are no data regarding expression of *finT* in *E. coli*, some preliminary data suggest that *finR* and *finA* are not well expressed in *E. coli* (data not shown), supporting the possibility that *finT* is poorly expressed in *E. coli*. A useful approach to the question of FinT expression in *E. coli* would be to use western blot analysis to compare FinT expression by *E. coli* bearing *finT*$^+$ plasmids and PA147-2. This would simultaneously address whether FinT can be made in *E. coli*, and how much is expressed relative to expression in PA147-2. However, even if FinT does mediate expression of genes in the 9kb region, downstream of *finT*, it seems unlikely that these genes would be involved in antifungal activity but have simply escaped the mutagenesis. To examine the problem further, it might be possible to generate maxicells using the previously constructed *recA* strain of PA109, bearing either a clone of the mutated or wildtype 16kb *Eco*RI region disrupted in PA109. If FinT has an influence on the expression of genes in the 16kb region, this should be readily observable by comparison of the proteins expressed by PA109 maxicells harbouring the mutated and wildtype 16kb

region. This is because PA109 has a mutation in *finT*, so expression of any FinT-dependent genes on the plasmid would result from the presence of *finT* in the wildtype clone, and would not be seen from the mutated clone. In addition, deletion of some or all of the 9kb region from the chromosome of PA147-2 would provide a definitive exposition of the involvement of the region in antifungal activity.

5.5.2 Analysis of a LysR-type transcriptional regulator.

DNA sequence analysis supports the identification of *finR* as a LTTR in a number of ways. The overall similarity between FinR and a number of other LTTRs is greater than 30%. In addition, the prediction of a LysR-type HTH motif in the N-terminus, the discovery of a T-N_{11}-A sequence with dyad symmetry in the intergenic region between *finR* and *finA*, and the regulation of a divergent gene all point toward a LTTR system. Experimental evidence to verify FinR as a LTTR would be the next logical step, and analysis of the DNA-binding properties of FinR in the intergenic region would be the most useful start point.

FINR138 IS DOMINANT WHEN PRESENT *IN TRANS.*

When the *finR138* allele was present in PA147-2 on a plasmid, a reduction in antifungal activity was observed. This result suggests that *finR138* is dominant over the wildtype allele, when over-expressed. Such an observation is consistent with current knowledge on LTTRs, which indicate that the functional form of LTTRs are homodimers or homotetramers (Schell, 1993). If FinR functions as a dimer or tetramer, the presence of multiple copies of *finR138* would likely lead to dimers or tetramers containing both functional and non-functional subunits, resulting in reduced or abolished functionality. The fact that antifungal activity was not completely abolished in these experiments suggests that either multimers containing both wildtype and mutant forms of the protein are somewhat active, or a subset of multimers are composed solely of wildtype protein. This basic experiment cannot distinguish between these explanations.

FINR AFFECTS *FINA* WHEN PRESENT *IN CIS.*

Investigations into the possible regulation of *finA* by FinR revealed that FinR had minimal impact on FinA expression when *finR* was *in trans*, relative to the effect of *finR138 in*

trans. However, when expression from a *finA*::*lacZ* fusion was measured from constructs with either *finR* or *finR138* present *in cis*, it became clear that the presence of *finR* has a significant regulatory effect on expression of FinA, and that replacing *finR* with *finR138* in PA138 probably results in dramatically reduced FinA expression.

The observation that *finR* was required *in cis* to affect *finA* is intriguing. The divergent arrangement of regulator and target genes is a common feature of LTTR systems (Schell, 1993), and a *cis* requirement might be the result of co-evolution of the system. In experiments on the *ilvYC* operon of *E. coli*, it was found that the genes are transcriptionally coupled (Rhee et al., 1999). Specifically, activity of the *ilvC* promoter was shown to be influenced by the transcriptional activity of the regulator *ilvY*. This was suggested to result from increased local supercoiling of DNA as a result of *ilvY* transcription, which increased activation of the superhelix-sensitive *ilvC* promoter (Rhee et al., 1999). The observation of transcriptional coupling provides supporting evidence for the suggestion that expression of the regulated gene is in part dependent upon the presence and location of the regulator gene, in addition to the DNA binding activity of the regulator protein. The ability of LTTRs to regulate expression of unlinked genes (Schell, 1993) could be a relatively new function of LTTRs (in evolutionary terms), not dependent upon genetic linkage. In other words, it is tempting to speculate that although LTTRs may have co-evolved with a linked target gene, it is possible some were recruited as regulators of unlinked genes at a later stage, with no requirement for linkage.

WHAT IS THE ROLE OF FINR IN FUNGAL INHIBITION?

It seems clear from the experiments described that *finR* is required for *in vitro* inhibition of fungal growth. A deletion of *finR* lacks the antifungal phenotype, as does the Tn*5* mutant PA138. Investigation of FinA regulation revealed that when *finR* is present *in cis*, there is greater than five-fold increase in FinA compared to the situation with the *finR138* allele present. However, when *finR* is absent (deleted), FinA appears to be expressed at intermediate levels. Furthermore, an insertion in *finA* did not abolish fungal inhibition, but reduced it. These data show that *finA* is required for full antifungal activity, but is not responsible for complete antifungal activity. Also, although the expression studies were plasmid based, the constructs mimic the genomic arrangement such that it might be

expected that some *finA* expression would occur in PA147-2*finR*Δ, leading to the conclusion that while *finA* plays some role in antifungal activity, low levels are insufficient to cause observable fungal inhibition. Therefore, besides its role in the regulation of *finA*, FinR most probably regulates additional, as yet uncharacterised, antifungal genes as discussed below.

WHAT IS THE ROLE OF FINA IN FUNGAL INHIBITION?

In addition to identifying *finR* as a probable LTTR of antifungal activity, study of PA138 and the *finR* locus identified a putative flavoprotein reductase gene (*finA*) that has a role in fungal inhibition. While there is considerable data in the literature regarding the substrates of such enzymes, and the reactions they catalyse, there is no evidence that any flavoprotein reductase enzymes have previously been implicated in antifungal compound synthesis. Thus, the demonstration of a role for FinA in fungal inhibition is a novel finding. However, although FinA has been shown to be important, no role for FinA can currently be assigned, due to the fact that the structure of the compound or compounds produced by PA147-2 has remained elusive. If a structure could be solved, it would then be possible to construct a testable pathway for the synthesis of the compounds, and a role for FinA might then be forthcoming. This has not yet been possible, due to the difficulty involved in preparing large, pure samples of inhibitory compound, and the apparent instability of antifungal extracts from PA147-2 (Godfrey, 1997). The synthesis of polyketide antibiotics (e.g. pyoluteorin, 2,4-diacetylphloroglucinol) requires a ketoreductase, either as a domain of a polyketide synthase or as a separate protein that forms part of a enzyme complex (Bender et al., 1999). One speculative suggestion is that FinA is in some way involved in synthesis of a polyketide, but since FinA has no resemblance to any known ketoreductase this seems unlikely.

5.5.3 FinT and FinR regulate common proteins.

As an approach to confirming that FinT and FinR encode global regulators, protein profiles of PA147-2, PA109, and PA138 were compared after growth in conditions that support antifungal compound production. In addition to examining total protein, *de novo* protein synthesis was examined by labelling proteins with ^{35}S-methionine and ^{35}S-cysteine for one

hour prior to SDS-PAGE and autoradiography. Examination of both the total protein and the *de novo* synthesised protein suggests that there are differences between PA147-2 and the mutants, and that some of these differences are common to both mutants. These results may represent an under-estimation of the differences between PA147-2 and the regulatory mutants because of the low resolution of the 1D analysis, and the fact that membrane-bound and secreted proteins would have escaped detection in these experiments. The differences in protein profiles supports the suggestion that both *finR* and *finT* encode global regulator genes, but does not reveal whether the regulation occurs at the level of transcription or translation. Two further approaches to the question of global regulation would be revealing in this regard. Firstly, it would be useful to build upon the one-dimensional SDS-PAGE study, and carry out two-dimensional PAGE. The use of 2-D PAGE would greatly increase the ability to detect differences in protein profiles between PA147-2 and the regulatory mutants, as its resolution is far superior to 1-D SDS-PAGE. A second and complementary approach would be RNA subtractive hybridisation, which would provide a means by which the degree of transcriptional regulation can be assessed, relative to that occurring at the post-transcriptional level. There is sufficient reason to consider both options for the future study of PA147-2. Using DNA and protein sequencing, differentially regulated genes can be identified. Construction of knockout mutants would then allow the role of these genes in fungal inhibition to be assessed. The probability of success is high, given that 1-D SDS-PAGE has already allowed differential gene expression to be visualised. The comparison of differentially regulated genes revealed by subtractive hybridisation with those found by protein profiles might allow the level of regulation of particular genes to be assessed. This could be very informative given the recent suggestion of a post-transcriptional mechanism for GacA (Blumer et al., 1999) in addition to its role as a transcriptional regulator (Chancey et al., 1999).

The observation that FinR and FinT are required for the expression of common proteins indicates a relationship between FinR and FinT. While the nature of the relationship remains unknown, preliminary data (not shown) on expression of *finT*::*lacZ* and *finR*::*lacZ* fusions in PA147-2, PA109, and PA138 indicate that expression of *finT* is independent of *finR*, and vice versa. While these data need to be confirmed, they point to a model in which FinR and FinT interact, perhaps in a synergistic fashion, to control the expression of a

number of genes. The growing picture of regulatory cascades indicates that phenotypes such as virulence and antifungal activity are characterised by a number of interacting regulators, which provide fine control over the various individual traits that contribute to the overall phenotype (Blumer & Haas, 2000b; Haas et al., 2000; Schell, 2000).

The experiments outlined in this chapter have shown that FinR has a role in the regulation of *finA*, a gene that is required for full expression of antifungal activity. In addition, 1D SDS-PAGE analysis of the mutants PA138 and PA109 provided evidence that a number of proteins are regulated by FinR and FinT. The identification of one target protein was attempted by N-terminal sequencing, but database searches failed to find any matching sequences. Despite the inability to identify the protein based upon its sequence, elucidation of the N-terminal sequences of the proteins with altered expression in the regulatory mutants may provide an avenue for further investigation. By designing degenerate primers based on the N-terminal sequence and carrying out PCR amplification with the degenerate primer and a universal primer, it may be possible to clone some or the entire gene encoding that protein. In combination with 2D protein electrophoresis, such an approach might facilitate the discovery of genes regulated by FinR and/or FinT. Establishing a role for that gene in fungal inhibition could then be accomplished by a site-directed mutagenesis approach and once the gene had been isolated, more quantitative techniques could be used to formally demonstrate regulation by FinR, FinT, or both proteins. Another useful approach would be to generate mono-specific polyclonal antibodies for each protein that is shown to be differentially expressed between the mutants and wildtype. Such antibodies could be used in westerns to show whether expression of the protein in question is abolished, reduced, or has migrated at a different rate due to altered post-translational modification in the mutants. Further studies using westerns could assess the effect of altered nutrient levels on protein expression in the wildtype, which would be interesting given the importance of minerals and carbon source in the expression of a number of known antifungal metabolites (Duffy & Defago, 1999; James & Gutterson, 1986; Slininger & Jackson, 1992).

Chapter 6

Involvement of the phosphate specific transport system in antifungal activity

6.1. Introduction.

6.1.1 The phosphate specific transport system.

The phosphate specific transport (Pst) system mediates high affinity uptake of inorganic phosphate under phosphate limiting conditions (Wanner, 1996). The genes encoding the Pst system are arranged in an operon, and in most cases are organised as shown in Figure 6.1.

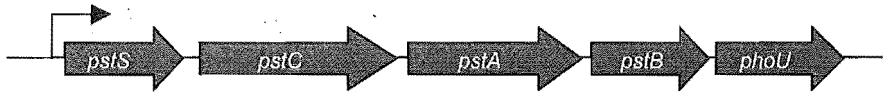


Figure 6.1. Organisation of the genes encoding the Pst system. The black arrow indicates the start of transcription.

Phosphate transport.

The Pst system is a transport complex belonging to the ABC transporter superfamily, and as such has a number of conserved features (Higgins, 1992). PstA and PstC are membrane spanning proteins, which associate to form a channel for Pi transport. PstB is an ATPase (Chan & Torriani, 1996) which forms a dimer that interacts with PstA and PstC on the cytoplasmic side of the inner membrane. PstB is thought to hydrolyse ATP providing the energy for Pi uptake, and PstS is located in the periplasm where it functions as a Pi binding protein, directing Pi to the transport complex. The Pi uptake system is illustrated in Figure 6.2. PhoU is not required for Pi uptake, but is essential for the regulatory functions in which the Pst complex is involved (Steed & Wanner, 1993). *E. coli* is known to have a number of other phosphate uptake systems, including a low affinity Pi transport (Pit) system, and transporters for organophosphates (Argast et al., 1978; Rao & Torriani, 1990; Wanner, 1996), and it is likely that *P. aeruginosa* also has a phosphate uptake system in

addition to Pst (Nikata et al., 1996). However, the Pst system is the main mechanism for scavenging inorganic phosphate under Pi limiting conditions in *E. coli* and *P. aeruginosa*.

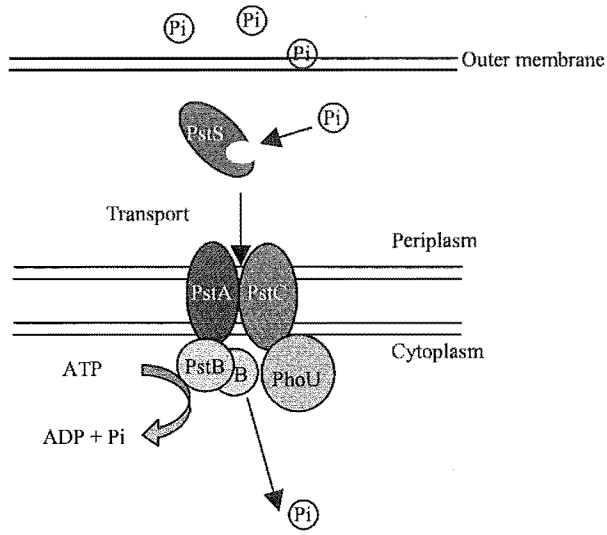


Figure 6.2. Diagram illustrating the current model for Pi uptake by the Pst complex. PhoU has no discernible role in Pi uptake, but is part of the Pst complex, having a regulatory role (see text for details).

Expression of the Pst operon is controlled largely by extracellular Pi concentration. The Pst complex is part of a regulatory network called the Pho regulon. In response to low extracellular Pi, expression of the genes in the Pho regulon is induced. There are at least 38 genes in the Pho regulon, including the *pst* genes, and for the most part these encode products involved with Pi transport and metabolism (Rao & Torriani, 1990; Wanner, 1995). Activation of the genes in the Pho regulon is controlled by PhoB, the response regulator of the two-component pair PhoR/PhoB. Phosphorylation of PhoB by the sensor kinase PhoR allows PhoB to activate transcription of Pho regulon genes by binding to a consensus Pho box within the promoters of target genes (Wanner, 1995; Wanner, 1996).

Control of the Pho regulon.

In addition to its role in high affinity Pi uptake, a membrane integrated Pst system also plays a pivotal role in the repression of the Pho regulon in high Pi environments. Evidence for this function comes from studies showing that certain mutations in *pst* genes result in loss of Pi uptake and derepression of the Pho regulon (Cox et al., 1989; Steed & Wanner, 1993). Loss of PhoU had no discernible impact on Pi uptake, but resulted in derepression

of the Pho regulon (Steed & Wanner, 1993). Additional evidence supports a role for Pst in both Pi uptake and Pho regulon repression, and also indicates that these two functions can be independent. Site directed mutagenesis was used to create mutations in *pstA* and *pstC* that abolished high affinity Pi uptake, but had no impact on Pho regulon expression (Cox et al., 1988; Cox et al., 1989). These mutations altered the membrane spanning proteins PstA and PstC, making them incapable of Pi transport. Presumably the overall structure of the complex remained unchanged, a feature which may be responsible for the continued ability to repress the Pho regulon.

Exactly how the Pst system and PhoU repress the Pho regulon is not well understood. The current model suggests that the sensor kinase PhoR is closely associated with the Pst complex, and this association is mediated via PhoU (Figure 6.3). Under high Pi conditions, a so-called "repression complex" forms in which occupation of Pi binding sites in the Pst complex (and possibly PhoR) lead to a change in PhoR conformation, inhibiting its ability to transfer phosphate to PhoB. Since PhoB is not phosphorylated under these conditions, the Pho regulon is repressed. The low Pi found in phosphate limiting environments results in low occupation of Pi binding sites, releasing PhoR from the "repression complex". In this situation, PhoR phosphorylates PhoB, thus derepressing the Pho regulon (Wanner, 1995).

The Pst system is expressed as part of the Pho regulon in response to Pi limitation, so PhoB activation mediated by PhoR release from the "repression complex" leads to higher levels of *pst* operon expression, and thus more Pi uptake. Although the Pst system and PhoU are essential for Pho regulon repression, they are only synthesised at a high level once repression has been removed. Seemingly a basal level of Pst system activity is sufficient to repress the Pho regulon under high Pi conditions.

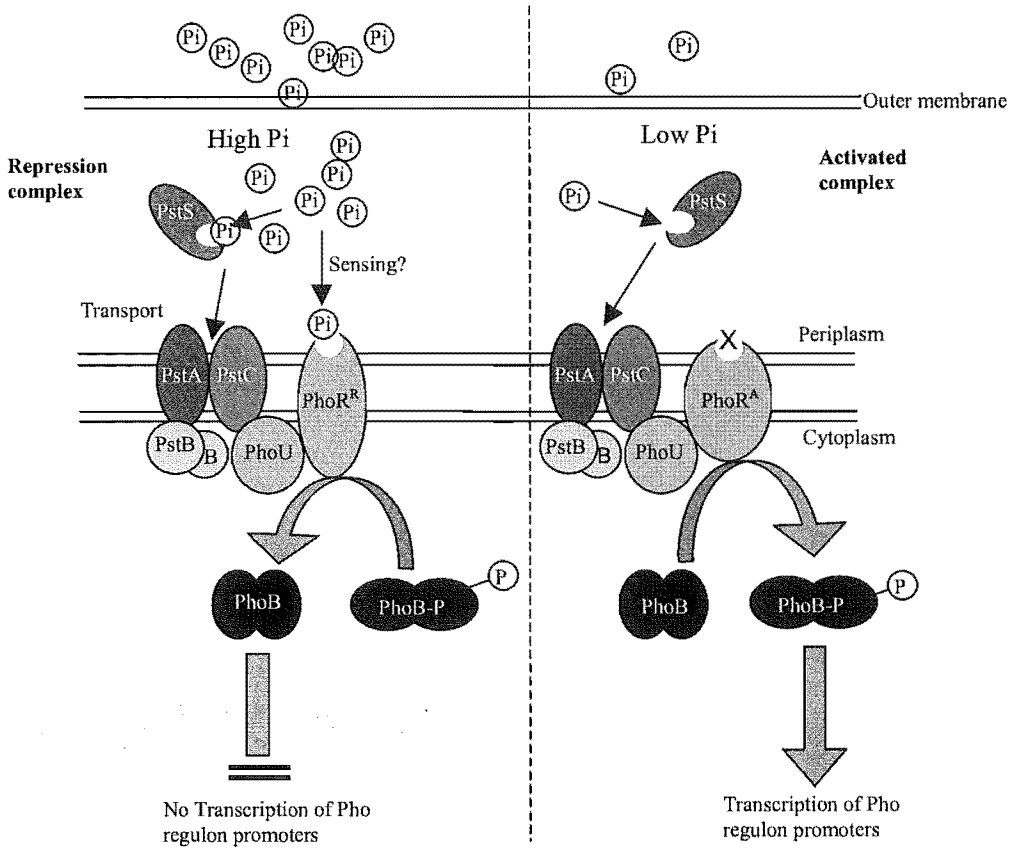


Figure 6.3. Model for the involvement of the Pst system and PhoU in repression of the Pho regulon (adapted from Wanner, 1996).. High Pi (left) results in formation of a repression complex, and PhoB cannot be phosphorylated. Low Pi (right) releases PhoR from the repression complex, causing PhoB phosphorylation, and thus transcription of Pho regulon genes. $PhoR^R$= PhoR repression due to high extracellular (and periplasmic) Pi. $PhoR^A$= PhoR is activated in response to low Pi. The exact nature of the repression complex is unclear.

Pst systems in other organisms.

The paradigm outlined above summarises findings regarding the Pst system in *E. coli*. To what degree are Pst systems conserved among diverse prokaryotes? Pst systems have been identified in a number of species including *Salmonella enterica* (Typhimurium) (Jiang et al., 1995), *Bacillus subtilis* (Takemaru et al., 1996), *P. aeruginosa* (Nikata et al., 1996), *P. putida* (Wu et al., 1999), *Caulobacter crescentus* (Gonin et al., 2000), and *Streptococcus pneumoniae* (Novak et al., 1999). With a few exceptions, initial studies seem to suggest that the organisation of the Pst genes, and the functions of the Pst system in these organisms are highly conserved.

6.1.2 Involvement of the Pst system with complex traits.

In addition to defects in Pi transport and Pho regulon control, a number of complex traits appear to require an intact Pst system. These traits would not intuitively be linked with the Pst system, and their dependence upon Pst indicates a role for the Pst system as an environmental sensor allowing diverse traits to be switched on or off depending on the extracellular Pi concentration. Although the Pst system directly senses periplasmic Pi levels, this can be taken as an indirect system to sense extracellular Pi, since Pi freely enters the periplasm from the extracellular milieu (Rao & Torriani, 1990). Three examples are outlined here.

PATHOGENICITY OF AN *E. COLI* THAT CAUSES SEPTICEMIA IN PIGS.

E. coli strain 5131 is serum-resistant and causes septicemia in infected pigs. A mutant screen was conducted with the aim of isolating strains with reduced pathogenicity. One such mutant was found to have a transposon insertion in the gene *pstS*, and was shown to be avirulent in chickens and pigs, and serum sensitive (Daigle et al., 1995). Expression of the Pho regulon by the wildtype appeared to have no effect on serum resistance, and the authors suggest that the avirulent phenotype is not related to Pho regulon expression. Rather, they propose that virulence is modulated directly by Pst via an unknown mechanism, or indirectly by Pst-mediated regulation of a gene or genes not linked to the Pho regulon.

INVASION GENE EXPRESSION IN *SALMONELLA ENTERICA* TYPHIMURIUM.

A screen for *S. enterica* serovar Typhimurium mutants that were reduced in their ability to express *hilA* and invasion genes identified *pstS* as being important for *hilA* expression (Lucas et al., 2000). HilA activates invasion gene expression, and a transposon insertion in *pstS* reduced *hilA* expression 2-3 fold. Construction of a *pstS-phoB* double mutant (cannot express the Pho regulon) suppressed the effect of the *pstS* insertion on *hilA* expression, indicating that the reduced *hilA* expression in the *pstS* mutant was due to an increase in Pho regulon activity. These authors suggest that using the Pho regulon to regulate invasion allows *S. enterica* to sense its location. They speculate that the site for invasion (lumen) is high in Pi, allowing expression of the invasion genes, while other environments (such as

the interior of a macrophage) are low in Pi, leading to repression of invasion genes, and expression of other genes required for survival (Lucas et al., 2000).

STALK ELONGATION IN *CAULOBACTER CRESCENTUS.*

Caulobacter crescentus produces morphologically distinct progeny as a result of asymmetric cell division. One cell (the swarmer) has a polar flagellum, while the other has a polar stalk. Environmental phosphate has a dramatic effect on stalk length – low Pi concentrations lead to elongation of the stalk by as much as 30 times, and it is thought that this allows *C. crescentus* to uptake Pi more efficiently. To determine the underlying mechanism, mutants were generated that produced elongated stalks irrespective of Pi concentration. A number of mutants were found to have insertions in the *pst* genes, and to examine the possibility that constitutive Pho regulon expression caused the stalk elongation, PhoB mutants were made. The results indicate that PhoB is required for stalk length, and this requirement is likely to involve PhoB-mediated expression of the Pho regulon (Gonin et al., 2000).

6.1.3 Phosphate and antibiotic expression.

The production of a number of antibiotics has been shown to be inhibited by high phosphate concentrations. These include candicidin and tetracycline production by *Streptomyces* spp., and vancomycin fermentations (Martin & Demain, 1980). Of particular relevance to biocontrol is the observation that phosphate can inhibit phenazine production by some (but not all) phenazine producing bacteria (Turner & Messenger, 1986), and that synthesis of 2,4-diacetylphloroglucinol and pyoluteorin by *P. fluorescens* CHA0 is reduced when phosphate concentrations are high (Duffy & Defago, 1999).

6.1.4 Objectives of this study.

The antifungal mutant PAH26 was described in chapter 4. DNA sequence analysis indicated that this mutant had an insertion in a homologue of *pstA*. Interestingly, in an independent study, a *pstC* mutant (PAB108) was isolated on the basis of a biofilm-defective phenotype (Monds, 2000). The main objective of the work presented was to examine the impact of a *pstA* mutation on antifungal activity, with a view to development

of a testable model. To accomplish this, it was necessary to undertake preliminary studies on Pho regulon control and Pi uptake by *P. aureofaciens*. The objective of the Pi uptake and Pho regulon studies was to develop an understanding of the impact of *pst* mutations on *P. aureofaciens*, which would then allow the design of experiments to examine the antifungal nature of the *pstA* mutant.

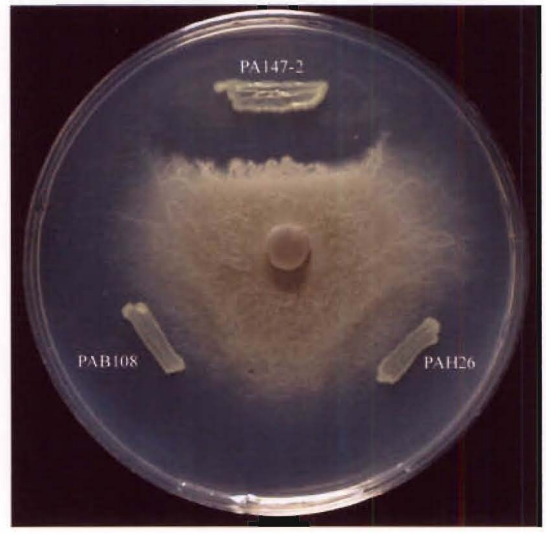
In this chapter the investigation into Pho regulon control and Pi uptake by PA147-2 and *pstA* and *pstC* mutants is presented. These data is used to design experiments to assess the cause of the antifungal defective phenotype. Speculative models regarding the role of Pst in antifungal activity are outlined, along with possible experimental approaches to test them. Finally, the novel aspects of Pi uptake and Pho regulon control by PA147-2 are discussed. Because the theme of this thesis is biocontrol, limited consideration is given to the biofilm-defective phenotypes. A manuscript detailing the biofilm results and aspects from this study has been submitted to Molecular Microbiology for publication.

6.2 Results.

6.2.1 PAH26 and PAB108 are antifungal- and biofilm-defective.

The DNA sequences derived from the mutants PAH26 (chapter 4) and PAB108 (Monds, 2000) indicated that both isolates have insertions in genes whose predicted translation products have a high degree of similarity to PstA and PstC (respectively) from *P. putida* and *P. aeruginosa*. Since the genetic basis of the Pst system is well conserved, it was predicted that the transposon insertions in PAH26 and PAB108 would not only interfere with expression of *pstA* and *pstC* respectively, but they would have polar effects on the expression of the downstream genes of the *pst* operon. Thus, it was reasoned, the insertions could lead to similar phenotypic effects. For this reason PAH26 was tested for its biofilm formation, and PAB108 was tested for the ability to inhibit *in vitro* growth of *G. graminis* var. *tritici*. Consistent with predictions, PAH26 did not form biofilms on abiotic surfaces, and PAB108 was unable to inhibit fungal growth (Figure 6.4).

a.



b.

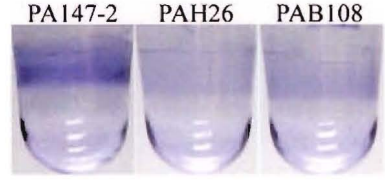


Figure 6.4. Phenotypic properties of PA147-2 and derivatives. **a**. Antifungal phenotype of PA147-2, PAB108, and PAH26 on tris-buffered PDA. A clearing zone between bacteria and fungus (*Gaeumannomyces graminis*) indicates inhibition of fungal growth. Both *pst* mutants are defective for fungal inhibition. **b**. Biofilm phenotypes of PA147-2, PAB108, and PAH26. Biofilm formation is visualised by staining with crystal violet. Biofilm formation is indicated by a ring of stained bacteria. Both *pst* mutants are defective for biofilm formation.

Further DNA sequencing was undertaken to confirm that the PA147-2 *pstA* was found in a similar arrangement to that in *P. putida*. DNA sequence was obtained from the ends of the clones pCMH26E and pCMH26. The sequences indicated that upstream of *pstA* were sequences with similarity to *pstC* and *pstS* (Figure 6.5). While not providing conclusive evidence that the *pst* genes of PA147-2 are organised identically to other organisms, these sequencing results indicated that at least three genes are in the anticipated configuration.

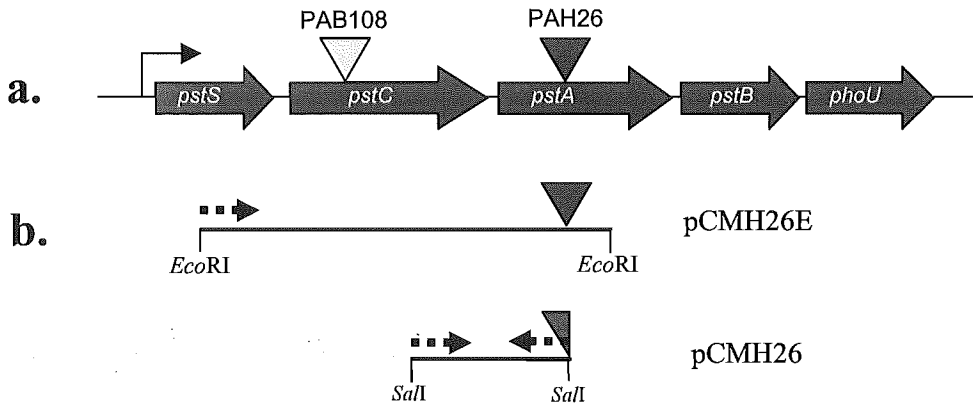


Figure 6.5. Cloning the Tn*5* insertion point from PAH26. **a.** Arrangement of *pst* genes in *E. coli* (Rao & Torriani, 1990) and *P. putida* (Genbank accession number AB017356), with transposon insertion points in PAB108 and PAH26 indicated. **b.** Clones pCMH26E and pCMH26, aligned with the *pst* genes in a. Regions of DNA sequence data derived from these clones are indicated by the broken arrows.

6.2.2 The physiological effects of *pst* mutations in *P. aureofaciens*.

Based upon the published analyses of *pst* mutants of *E. coli* and *P. aeruginosa* (Nikata et al., 1996; Steed & Wanner, 1993), PAH26 and PAB108 were predicted to have two physiological traits related to involvement in high-affinity Pi assimilation and Pho regulon control. Firstly, *pst* mutants have severe defects in high-affinity Pi uptake under Pi starvation conditions, and secondly, *pst* mutants show constitutive derepression of the Pho regulon. These phenotypes were examined in PAH26 and PAB108 in order to gain an understanding of the basic consequences of *pst* mutations in *P. aureofaciens*, so that predictions could be made as to why insertions in *pst* genes render PA147-2 antifungal defective. In experiments that use media with defined Pi concentrations, the stated inorganic concentration represents that Pi which was added. The presence of contaminating Pi from other sources was not ruled out, but was deemed to be of low

importance because phenotypic traits were always examined to determine whether a medium was limiting or sufficient for Pi.

PAH26 AND PAB108 HAVE DEFECTS IN PHO REGULON CONTROL.

A membrane integrated Pst system is required to repress expression of the Pho regulon under Pi sufficient conditions. Thus, it was predicted that both PAH26 and PAB108 would show constitutive Pho regulon expression, since PstA and PstC are both required for a functional Pst complex. The gene for alkaline phosphatase (APase) is a member of the Pho regulon in *E. coli*, and is often used as an indicator of Pho regulon activity (Wanner & Wilmes-Riesenberg, 1992) because its expression is relatively easy to assay. The effect of the *pstA* and *pstC* mutations on Pho regulon control was initially examined in a qualitative manner, by streaking PA147-2 and the *pst* mutants onto K10P agar plates (see appendix 1). K_2HPO_4 was added to give final calculated phosphate concentrations of 0.1, 1, and 25mM, and the plates were supplemented with BCIP. K10P plates were used because it is known that 10% King's B (K10) with the standard Pi concentration supported both antifungal activity and biofilm formation. In the presence of 0.1mM Pi, PA147-2 and the mutants were all blue, indicating APase (and Pho regulon) expression. When 1mM Pi was used, the mutant colonies were still blue, but the wildtype was not. On 25mM Pi plates, all strains showed negligible APase activity. The lack of APase expression by the *pst* mutants when grown on 25mM Pi was unexpected since studies of other organisms report that the Pho regulon in *pst* mutants is constitutively derepressed.

Quantitative assays for Pho regulon (APase) activity were carried out as described in chapter 2. The compound p-nitrophenol phosphate (pNPP) is cleaved by APase releasing a yellow compound (p-nitrophenol). Spectrophotometric measurement of the change in absorbance at OD_{410} over time allows the quantitative determination of APase activity for strains grown in the presence of different Pi concentrations. PA147-2, PAH26, and PAB108 were grown for 16 hours in K10P supplemented with K_2HPO_4 to give final Pi concentrations of 0.1, 0.5, 5, 10 and 25mM. Consistent with predictions, APase activity was comparably high for all strains when grown in Pi-limiting conditions (0.1mM Pi). When the Pi concentration was 0.5mM, PAH26 and PAB108 still showed high APase activity, while PA147-2 showed very low APase activity, indicating repression of the Pho

regulon. However, as Pi concentration was increased to 5, 10, and 25mM, both of the *pst* mutants demonstrated progressive reductions in APase activity. Although both *pst* mutants have defects in Pho regulon control, they both show some ability to repress the Pho regulon if extracellular Pi concentrations are high enough. Further to this, it is interesting to note that the *pstC* mutation appears to have a more adverse affect on Pho regulon control than the *pstA* mutation (Figure 6.6).

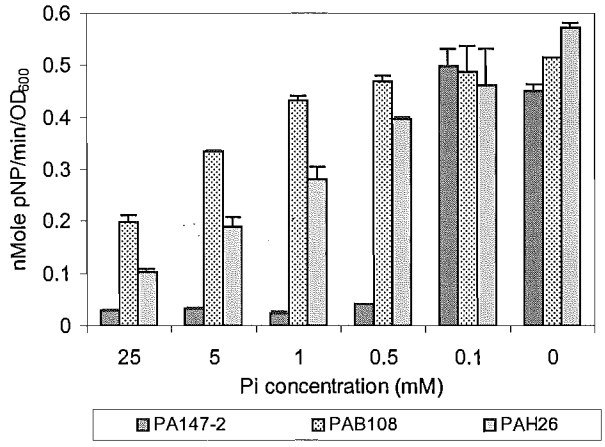


Figure 6.6. APase activity of PA147-2, PAH26, and PAB108 after 16 hours growth in Pi limiting, and excess conditions (K10P made with the stated Pi concentrations).

Alkaline phosphatase control experiments with *P. aeruginosa*.

Since the APase experiments with *P. aureofaciens* yielded some surprising results, control experiments were performed using *P. aeruginosa* PA01 (wildtype) and NT1 (*pstCAB-phoU* deletion) (Nikata et al., 1996). APase assays were carried out on both strains following 16 hours of growth in K10P-0.1, K10P-1, and K10P-25 media. As expected, PA01 showed APase expression after growth in 0.1mM Pi, but negligible APase after growth in 1 and 25mM Pi. Also consistent with expectations, NT1 had a high level of APase after growth in 0.1 and 1mM, indicating loss of Pho regulon control. Intriguingly, after growth in 25mM Pi, the APase level was reduced (Figure 6.7). This is discussed further in section 6.3.2.

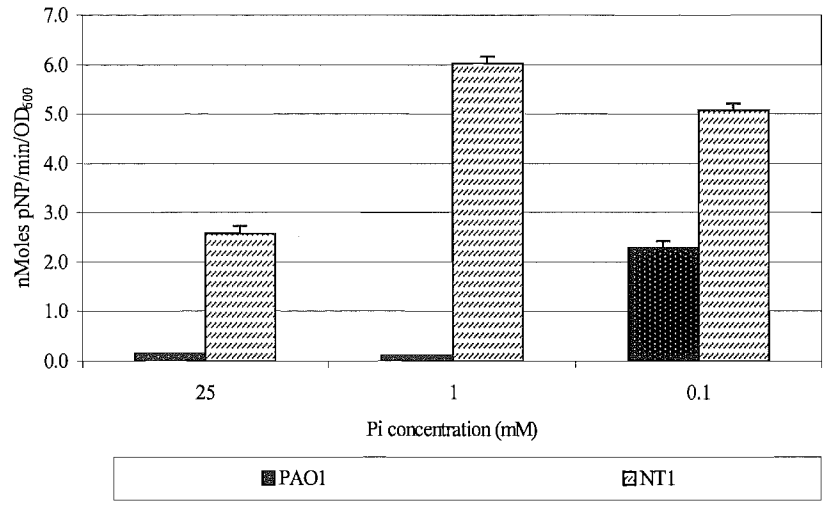


Figure 6.7. APase assays for *P. aeruginosa* PA01 and NT1 grown in the presence of 0.1mM, 1mM, and 25mM inorganic phosphate. APase is derepressed in PA01 grown in 0.1mM Pi, but repressed at the higher Pi concentration. Data were averaged from three replicates, and are shown with standard errors.

PI UPTAKE IS DEFECTIVE BUT NOT ABOLISHED.

Since a major function of the Pst system is high affinity uptake of Pi under Pi-limiting conditions, it is reasonable to expect that mutations in *pst* genes would result in abolition of high-affinity Pi uptake. Indeed, this has been the subject of extensive study in *E. coli* and *P. aeruginosa*, and *pst* mutants of both species are incapable of high affinity Pi uptake (Cox et al., 1988; Cox et al., 1989; Nikata et al., 1996; Rao & Torriani, 1990). Similar results were predicted for PAH26 and PAB108 since the mutations in these strains are thought to lie in *pstA* and *pstC*, the genes encoding the transmembrane proteins of the Pst system. To test Pi transport in PAH26 and PAB108, Pi uptake studies using $[^{32}P]K_2HPO_4$ were carried out for strains grown in Pi-limiting and excess conditions. Two strains of *P. aeruginosa*, PA01 and NT1 were included as controls.

Pi uptake under Pi excess conditions.

To determine the level of Pi uptake that is considered to be independent of the Pst system, initial measurements were made in Pi-excess conditions, in which PA147-2 should not be using high affinity transport. PA147-2, PAH26, and PAB108 were grown for 5 hours in

K10P-0.1. Following this, Pi uptake was measured at 30 second intervals for a three minute period. While K10P-0.1 is Pi limiting after 16 hours growth (Figure 6.6), APase assays showed that after only five hours, K10P-0.1 is still Pi-sufficient. This was demonstrated by the fact that PA147-2 had low APase activity under these conditions, relative to PAH26 and PAB108 (Figure 6.8).

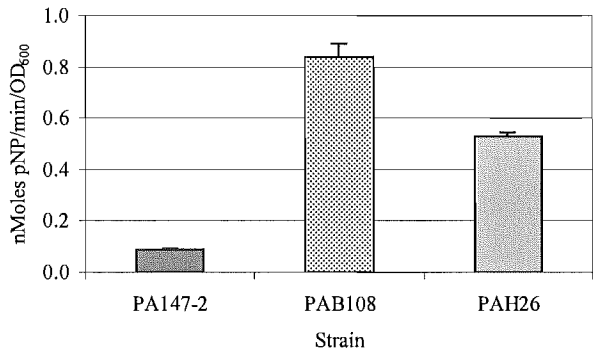


Figure 6.8. APase assays for PA147-2, PAH26, and PAB108 grown for five hours in K10P-0.1. The low APase activity of PA147-2 indicates Pho regulon repression, suggesting that Pi was not limiting in these cultures. Data are the average from three replicates. Standard errors are shown.

PA147-2 showed negligible Pi uptake under the Pi sufficient conditions, but contrary to expectations, PAH26 and PAB108 were able to assimilate a considerable amount of Pi, relative to the wildtype. Furthermore, PAB108 could uptake more Pi than PAH26 (Figure 6.9).

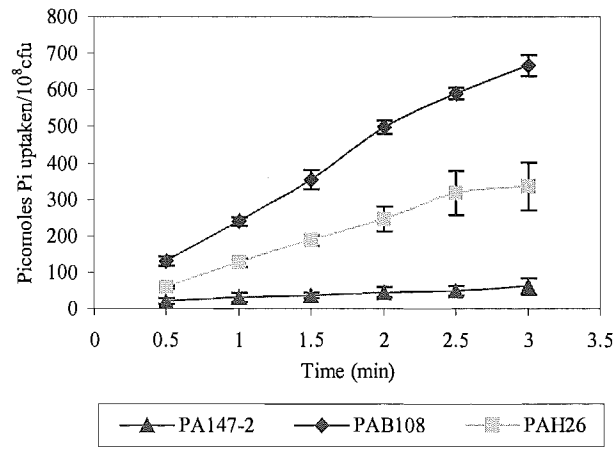


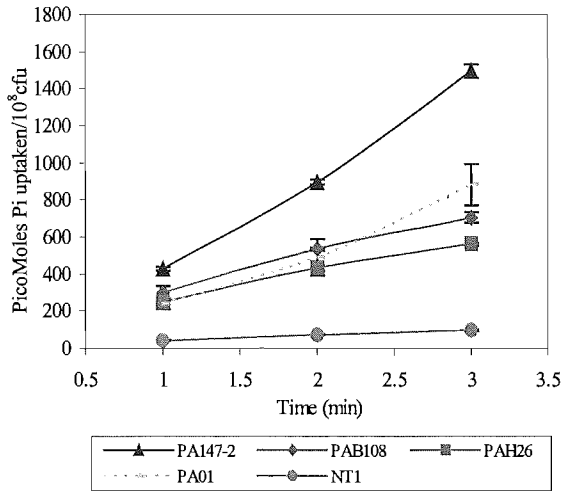
Figure 6.9. Pi uptake by *P. aureofaciens* strains grown for five hours in K10P-0.1. Although these are Pi sufficient conditions, PAB108 and PAH26 show considerable Pi uptake, compared to the very low Pi uptake by PA147-2. These data were averaged from three independent experiments, and standard errors are shown.

Pi uptake in Pi-limiting media.

Because of disruptions to the Pst complex, *pst* mutants are expected to have serious defects in their ability to assimilate Pi when grown under Pi limiting conditions. To test this assumption, Pi uptake by PAH26 and PAB108 was measured after growth for 20 hours in the Pi limiting medium $T_{0.05}$ (see appendix 1). *P. aeruginosa* PA01 and NT1 were included as controls. This was important given the unexpected results for Pi uptake in Pi sufficient conditions.

When grown in $T_{0.05}$ medium, PAH26 and PAB108 exhibited an approximately 50% reduction in total Pi uptake relative to PA147-2 (Figure 6.10). This was in stark contrast to *P. aeruginosa* NT1, which assimilated a negligible amount of Pi compared to PA01 and all three *P. aureofaciens* strains. PAB108 was able to assimilate approximately the same amount of Pi as it could in Pi-excess conditions, while PAH26 assimilated slightly more than under Pi-excess conditions. However, both mutants internalised less Pi than PA147-2, despite the observation that all three strains were expressing approximately equal levels of APase (indicating equivalent Pho regulon activity) Figure 6.10b). While this reduced Pi uptake ability is in agreement with the predicted affect of *pstA* and *pstC* mutations, it is clear that both strains were still capable of significant high-affinity Pi uptake (Figure 6.10a). The fact that the $T_{0.05}$ was limiting for Pi was confirmed by carrying out APase assays. PA147-2 and PA01 both showed expression of APase, indicating de-repression of the Pho regulon in response to Pi starvation (Figure 6.10b).

a.



b.

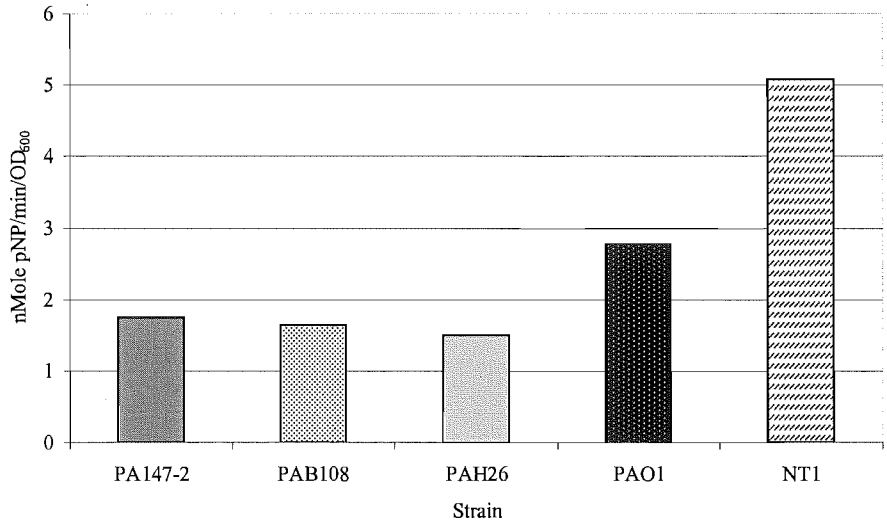


Figure 6.10. Pi uptake and alkaline phosphatase activity after growth in Pi limiting media. **a**. Pi uptake by *P. aureofaciens* and *P. aeruginosa* strains grown for 20 hours in $T_{0.05}$ medium. Only NT1 is completely defective for Pi uptake. **b**. APase assays carried out after 20 hours growth in $T_{0.05}$.

Pi-uptake after growth in excess Pi (5 and 25mM).

PAH26 and PAB108 demonstrated some ability to repress the Pho regulon when grown in excess Pi conditions (5 and 25mM). It was of interest to examine the effects of Pi excess on high-affinity Pi uptake – would this mirror the APase observations? PA147-2 and the

two *pst* mutants were grown for five hours in K10P-5 and K10P-25, after which Pi uptake assays were carried out. These results (Figure 6.11) demonstrate that as Pi increases in the growth medium, the ability of PAH26 and PAB108 to rapidly assimilate Pi is diminished. The implications of the observation are discussed in section 6.3.3.

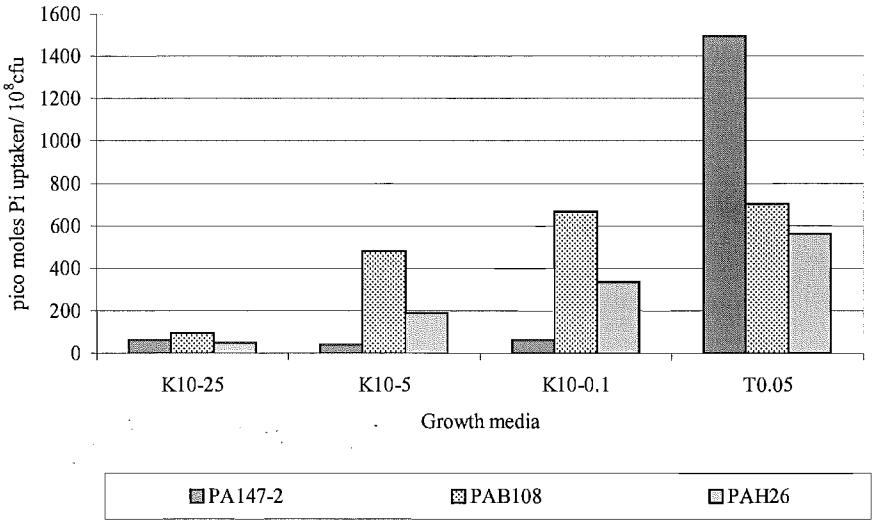


Figure 6.11. Cumulative Pi uptake by PA147-2, PAH26, and PAB108. *P. aureofaciens* strains were grown in Pi limiting and excess conditions, and the amount of Pi assimilated in three minutes was measured. Pi uptake ability is reduced for all strains with increasing Pi in the growth media.

6.2.3 How does a *pstA* mutation affect antifungal activity?

The experiments outlined above were undertaken to establish an understanding of how *pst* mutations affect the physiology of *P. aureofaciens*. Having established that PAH26 differs from PA147-2 in both Pho regulon control and Pi uptake, in a Pi-dependent fashion, the task was to ask whether these results had any significance to antifungal activity. Experiments were conducted to examine the following three possible explanations for the defect in fungal inhibition shown by PAH26.

1. The defect in high-affinity Pi assimilation might result in a loss of antifungal activity as a consequence of Pi starvation, and the induction of phosphate starvation induced (*psi*) genes.
2. Antifungal activity is subject to negative regulation by PhoB, or a member of the Pho regulon. Thus, the apparent loss of Pho regulon control in PAH26 would be the reason PAH26 is antifungal minus.

3. The Pst system might be required for the control of antifungal activity in low Pi conditions, via a mechanism not linked to PhoB/PhoR-mediated control of the Pho regulon. Thus, PAH26 is antifungal-defective because of the loss of the Pst complex, and not the high expression of Pho regulon genes in PAH26.

Is Pi starvation responsible for the Antifungal minus phenotype of PAH26?

Investigation of the high-affinity uptake of Pi by PAH26 revealed a defect in Pi assimilation under low Pi conditions. However, PAH26 was also shown to be capable of assimilating more Pi than the wildtype when the extracellular Pi concentration was sufficient. To test the role of Pi starvation, PAH26 was tested for antifungal activity on a minimal medium containing 0.5mM Pi. APase assays had previously shown that PA147-2 did not express the Pho regulon under such conditions, indicating they were Pi-excessive. Thus, it was predicted that if Pi starvation was responsible, the provision of 0.5mM Pi would overcome the starvation and restore antifungal activity. The result is shown in Figure 6.12. It is clear that PAH26 is still incapable of inhibiting fungal growth under these conditions.

Is Pho regulon expression responsible for the antifungal minus phenotype of PAH26?

Results from the investigation of biofilm formation by *pst* mutants indicate that expression of the Pho regulon negatively regulates biofilm formation (R. D. Monds, M. W. Silby, and H. K. Mahanty, manuscript submitted). To examine this possibility with respect to fungal inhibition, antifungal assays were carried out on minimal media supplemented with 0.05, 5, and 25mM Pi. It was predicted that if the Pho regulon had a negative effect on fungal inhibition, PA147-2 would be rendered antifungal minus on the 0.05mM Pi plate, while PAH26 would be restored in its ability to inhibit fungal growth when supplied with 5 or 25mM Pi. These predictions were made on the basis that 0.05mM Pi would derepress the Pho regulon in PA147-2, while 5 or 25mM Pi might sufficiently reduce Pho regulon expression by PAH26, as shown previously. As can be seen from the result in Figure 6.12, PAH26 was capable of inhibiting *G. graminis* var. *tritici* when provided with 25mM Pi. However, PA147-2 was still capable of fungal inhibition under Pi-limiting conditions, although it appears slightly reduced in this capacity.

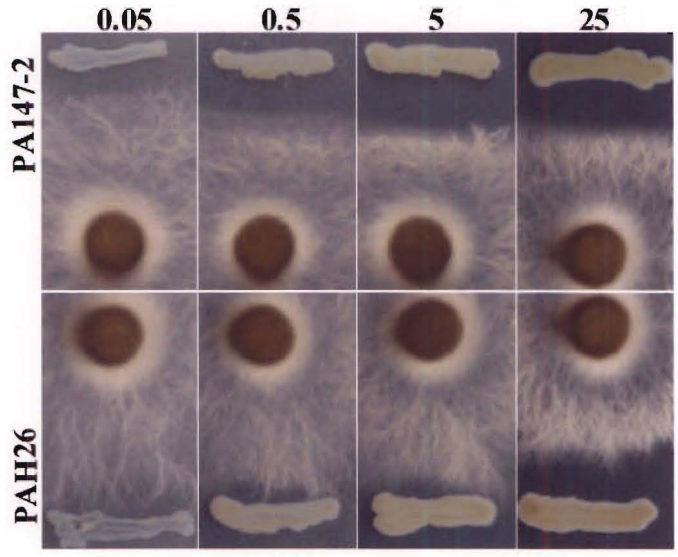


Figure 6.12. Fungal inhibition assays with in the presence of varied Pi concentrations. Bioassays were carried out against *G. graminis* var. *tritici*, on minimal bioassay medium with 0.05, 0.5, 5, and 25mM added Pi. PA147-2 is capable of fungal inhibition under all conditions, although the zone of inhibition in the presence of 0.05mM Pi appears to be smaller than that produced under Pi excess conditions. PAH26 is restored in its ability to inhibit fungal growth in the presence of 25mM Pi.

6.2.4 Can PA138 and PA109 be restored for antifungal activity by high Pi?

The experiment outlined above indicated that PAH26 could inhibit fungal growth on media containing high Pi concentrations, but the significance of this was not clear. The possibility existed that restoration of antifungal activity was common among the mutants, and not necessarily directly linked to the *pstA* mutation. To test whether this is a common trait amongst PA147-2 antifungal mutants, antifungal assays were carried out with PA138(*finR*$^-$) and PA109(*finT*$^-$) under low Pi and high Pi conditions. These experiments show that PA138 is defective for fungal inhibition under all conditions tested. However, PA109 showed signs of inhibition at 5mM Pi, and strongly inhibited *G. graminis* var. *tritici* in the presence of 25mM Pi (Figure 6.13). Therefore, restoration of antifungal activity in the presence of excess Pi is not unique to PAH26, but is also not a general feature of antifungal mutants of PA147-2.

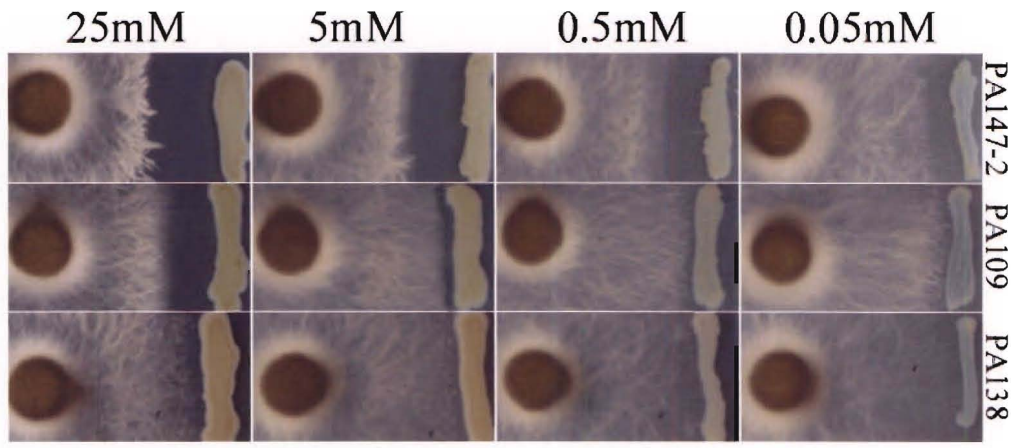


Figure 6.13. Fungal inhibition by *P. aureofaciens* PA147-2, PA109 and PA138 in the presence of varied Pi concentrations. The bioassays were carried out against *G. graminis* var. *tritici* on minimal bioassay media supplemented with 0.05, 0.5, 5, and 25mM Pi. PA138 is unable to inhibit growth of the fungus. PA147-2 is capable of fungal inhibition under all conditions tested. PA109 is restored for fungal inhibition in the presence of 25mM Pi, and shows limited inhibition in the presence of 5mM Pi.

6.2.5 Soluble protein profile of PAH26.

The Pst system is involved in the regulation of the Pho regulon, and the Pho regulon is differentially expressed in PAH26 relative to PA147-2. Thus, the possibility existed that the antifungal defect in PAH26 was the result of negative regulation of antifungal genes by a member of the Pho regulon, or an intact Pst system was required for expression of the antifungal genes. If the *pstA* mutation was directly or indirectly preventing the expression of a number of antifungal genes, the protein profile of PAH26 might resemble that of the regulatory mutants PA138 and PA109 (chapter 5). To examine this possibility, PAH26 was grown in buffered PDB for two days, and a sample was removed each day and incubated with Promix for one hour to allow incorporation of labelled methionine and cysteine into *de novo* expressed proteins. Total soluble protein was then separated by SDS-PAGE, and the profile was compared to that of PA147-2, PA138, and PA109. The protein pattern from PAH26 was indistinguishable from PA147-2, rather than showing differences such as those in the profiles of PA138 and PA109 (Figure 5.16). Autoradiography of the *de novo* expressed proteins confirmed the observations of total soluble protein. Thus it appears that the defect in PAH26 does not impact upon gene expression in the same way as the regulatory mutations.

6.3 Discussion.

In this chapter, the initial characterisation of phosphate-specific transport (*pst*) mutants of *P. aureofaciens* PA147-2 is reported. Two aspects of the results presented are novel; phosphate specific transport has not previously been associated with either fungal inhibition or biofilm formation, and *pst* mutants of *P. aureofaciens* have different characteristics compared to *pst* mutants of *E. coli* and *P. aeruginosa*.

6.3.1 The Pst system is involved in antifungal activity and biofilm formation.

Mutations in *pstC* and *pstA* have been shown to abolish fungal inhibition and biofilm formation by PA147-2. Given the role of the Pst system in other organisms, three possible explanations for the observations were forthcoming. Firstly, loss of the ability to inhibit fungi and form biofilms could result from a generalised reduction in intracellular phosphate concentration (i.e. phosphate starvation), given that both PstA and PstC are part of the membrane-spanning channel for high-affinity Pi uptake. A second possibility was that biofilm formation and antifungal activity could be repressed by the Pho regulon, which is expressed under both Pi limiting and sufficient conditions by *pst* mutants. Finally, Pst could regulate the antifungal and biofilm phenotypes by mechanisms unrelated to Pi uptake or metabolism.

PHOSPHATE STARVATION?

In *P. aeruginosa*, null mutations in the *pst* operon result in an inability to rapidly uptake Pi under phosphate limiting conditions (Nikata et al., 1996). However, the results presented here demonstrate that both PAH26 and PAB108 are still capable of assimilating Pi, and are thus unlikely to be experiencing abnormally low intracellular Pi concentrations. To separate Pi starvation from Pho regulon activity, an alternative phosphate source was sought. In *E. coli*, the organophosphates glycerol-3-phosphate (G3P) and glucose-6-phosphate (G6P) are imported by specific mechanisms unrelated to Pst (Argast et al., 1978; Winkler, 1966), and G3P does not repress the Pho regulon (Brzoska et al., 1994). Based upon these observations in *E. coli*, it was reasoned that if Pi starvation is responsible for the loss of fungal inhibition, providing G3P or G6P should restore activity. However, if Pho regulon activity is the cause of the antifungal defect, the organophosphates should have no

effect since they do not repress the Pho regulon. Unfortunately, this experiment failed to make the distinction between phosphate starvation and Pho regulon activity because preliminary APase assays revealed that the organophosphates could repress Pho regulon expression in *P. aureofaciens* (not shown). To test the possible role of Pi starvation, fungal inhibition assays were carried out using a medium containing 0.5mM Pi. APase assays on PA147-2 showed these conditions to have excess Pi, so the fact that antifungal activity is not restored in PAH26 under these conditions supports the conclusion that Pi starvation cannot adequately explain the antifungal phenotype of PAH26. The low APase expression by PA147-2 indicates that PA147-2 is not starved for Pi under such conditions, so *P. aureofaciens* strains should have sufficient access to Pi, independent of the high affinity uptake shown by PAH26.

PHO REGULON ACTIVITY?

The fact that PAH26 and PAB108 still display considerable Pho regulon activity when grown in Pi sufficient conditions allows the question of Pho regulon control to be considered independently of Pi starvation. Antifungal activity by PAH26 can be restored in the presence of 5mM Pi or higher. This observation correlates with the reduction in Pho regulon activity at high Pi concentrations. However, PA147-2 is still capable of inhibiting fungal growth when assayed on media containing only 0.1mM Pi, a concentration at which the Pho regulon is expressed by PA147-2. Thus, although there is a correlation between the restoration of antifungal activity and reduction in Pho regulon expression in PAH26, it is unlikely that Pho regulon-mediated repression of antifungal activity provides a suitable explanation for the antifungal defect of PAH26, since PA147-2 would be expected to be antifungal defective when expressing the Pho regulon. This is in contrast to the production of a biofilm, in that PA147-2 is unable to form biofilms under Pi-limiting conditions (Monds 2000). Furthermore, mutants with insertions in *phoR* are unable to express the Pho regulon, and these mutants are able to form strong biofilms in Pi-limiting conditions (R. D. Monds, M. W. Silby, and H. K. Mahanty, manuscript submitted). Hence, it seems that the Pho regulon negatively regulates biofilm formation, but probably not fungal inhibition.

PST-MEDIATED CONTROL OF FUNGAL INHIBITION.

The final possible explanation for the antifungal defective phenotype of PAH26 is that the Pst system is involved in fungal inhibition in a way unrelated to phosphate assimilation and metabolism. The defect can be overcome by high Pi, but the apparent correlation between reduced Pho regulon activity and restored fungal inhibition under these conditions may be merely coincidental – Pi could act to suppress the defect in some other, unknown way. A similar observation has been made in *Streptococcus pneumoniae* (Novak et al., 1999). Although a *pstB* mutant demonstrated inhibition of autolysis when challenged with penicillin (i.e. increased tolerance), Pi concentration had no effect on the penicillin tolerance of the wildtype. This indicates that PstB might be involved in a Pi-independent signalling pathway.

The observation that Pho regulon expression does not prevent antifungal activity by PA147-2, but that high Pi can restore antifungal activity in PAH26 separates regulation by the Pho regulon from the puzzle. The question that remains is how does the *pstA* mutation of PAH26 render the strain defective for fungal inhibition? Two speculative models are presented below. Both are consistent with current knowledge, and have explanatory power. Furthermore, both models are testable, allowing future investigators to examine the situation more closely.

Model one – alternative regulatory pathways.

A possible explanation for the observations regarding PAH26 is that there are two regulatory pathways that PA147-2 uses to synthesise the antifungal metabolite. Pathway one is dependent upon the Pst system, in a manner independent of Pho regulon expression. This model must include the requirement that the Pst-dependent system is independent of the Pho regulon because of the fact that PA147-2 can inhibit fungal growth while expressing the Pho regulon. In this model, increasing Pi would lead to repression of the Pho regulon, and therefore a reduction in the synthesis of the Pst complex, and therefore reduced Pst-dependent expression of the antifungal metabolite. The second regulatory pathway in this model would operate under excess Pi conditions, and may be under negative regulation by a member of the Pho regulon. By controlling the second pathway with the Pho regulon, a smooth transition between the two pathways could be assured --as

Pst levels decrease and pathway one (Pst dependent) ceases activity, pathway two would be released from the negative regulatory constraints of the Pho regulon, to take over from the first pathway.

The most obvious experimental test for this model is a mutant hunt. If the proposal is correct, it should be possible to isolate mutants with opposite traits to PAH26. These new mutants would demonstrate antifungal activity under low Pi conditions, but would be antifungal defective under excess Pi conditions. It is possible that some such mutants would have defects in the second regulatory pathway, such that when the Pst-dependent system discontinues its activities, there is no second pathway to replace it. The mutant hunt could be complemented by RNA subtractive hybridisation, to examine genes that are expressed at different Pi concentrations, and 2-D protein electrophoresis, which would allow differences to be detected at the level of translation. While neither of the latter two approaches would provide phenotypic information, they could be readily applied to the analysis of mutant PAH26 and any new mutants obtained in the testing of this model, aiding in the identification of regulator genes and their targets.

Clearly, the experimental approach outlined above is heavily dependent upon the successful isolation of mutants. If none were recovered, it would be difficult to draw solid conclusions. However, assuming a large number of candidates were screened, the failure to obtain mutants would reduce the likelihood of the model being correct, and alternatives would be sought.

Different antibiotics are made in different environments.

In order to account for the observations regarding PAH26, the second model is similar to the first in that it has two components, one of which is Pst-dependent but independent of the Pho regulon, and the second is important in excess Pi conditions. In this second model it is proposed that PA147-2 produces at least two antifungal compounds, and that their production is dependent upon Pi concentration. Under low Pi, one compound is produced in a Pst-dependent fashion, and the second is repressed, possibly by the Pho regulon. In Pi-excess conditions, the Pst-dependent antifungal metabolite is not produced due to very low

levels of the Pst complex, but the second compound is produced, possibly as a result of being removed from the regulatory repression of the Pho regulon.

This model could be tested in a similar manner to the first model, by conducting a hunt for mutants able to inhibit fungal growth on Pi-limiting media, but not on Pi-excess media, and vice versa. Such a mutant search would be likely to identify regulator genes (as for model one), but could also reveal genes specific for the synthesis of antifungal compounds. While biosynthetic genes have not been found to date, a mutant search under specific conditions might be fruitful since the knockout of a single metabolite could be observed. This is in contrast to searching for mutants that are defective under all conditions, which may have mutations in a gene required for the expression of a number of antifungal compounds. A complementary strategy to test this model would be to examine the chemical nature of inhibitory compounds produced under low and high Pi conditions. If crude extracts showed different properties this approach would lend support to the notion of different compounds. Purification of the inhibitory compounds would allow a definitive assessment of whether Pi concentration leads to the synthesis of different compounds, although such analyses have proven difficult due to instability of antifungal extracts (Godfrey, 1997).

The possibility that Pi concentration leads to the differential regulation of antibiotic expression is supported by the observation that production of Phl and pyoluteorin by *P. fluorescens* CHA0 is reduced in the presence of high Pi (Duffy & Defago, 1999). Furthermore, Duffy and Defago (1999) show that Phl production by a range of other *P. fluorescens* biocontrol strains is reduced by high Pi. Similar observations have been made regarding the production of antibiotics by *Streptomyces griseus* and *S. aureofaciens* in fermentation. Addition of greater than 10mM inorganic phosphate can repress antibiotic production (Martin & Demain, 1980). Since the mechanism for the repression by Pi is unknown, these observations could support both models outlined above. PA147-2 might make an antibiotic whose synthesis is repressed by high Pi, but also be capable of producing a second compound in the high Pi environment. Alternatively, Pi dependent repression could act on a regulator of antibiotic synthesis, preventing its expression in high Pi environments (see discussion below on the role of FinT). PA147-2 could circumvent this by use of an alternative regulator at high Pi concentrations.

Does the Pho regulon repress a Pi sufficient antifungal system?

Both of the models outlined above require that when Pi is limiting, there is repression of an antifungal system that is expressed under Pi-excess conditions, and it is suggested that a member of the Pho regulon could be involved in this repression. A simple test of this Pho regulon dependent repression would be to create null mutations in either *phoR* or *phoB* in PAH26. Since PhoB/R is the two-component regulator system that controls expression of the Pho regulon, the resulting mutants would be unable to express the Pho regulon. The prediction is that by abolishing Pho regulon expression, any antifungal systems normally repressed by the Pho regulon would be expressed, and PAH26 would be restored to full antifungal activity irrespective of Pi concentration.

A role for finT in Pi dependent fungal inhibition?

The observation that PA109 could inhibit fungal growth on high Pi media suggests that one of the two models above may approach the true situation. It is possible that FinT is required for fungal inhibition at low Pi, as part of the Pst-dependent system, while at high Pi concentrations the requirement for FinT is reduced because of the activity of a Pst-independent, high Pi system. The investigation into soluble protein profiles of PA109 and PA138 suggested some common regulatory targets (chapter 5). These experiments were conducted under low-Pi conditions, so it would be interesting to examine the profile of PA109 after growth in high Pi. It is also interesting to note that in the protein profile experiments, PAH26 did not show the absence of proteins that were missing in PA109. It is tempting to speculate that FinT regulates a number of genes for antifungal activity under low Pi, while the Pst complex has very specific effects on just one or a few genes/proteins.

A COMMON SWITCH FOR BIOFILM FORMATION AND FUNGAL INHIBITION?

It is intriguing that both fungal inhibition and biofilm formation are dependent upon an intact Pst system. Although the experiments outlined here and elsewhere (Monds 2000; R. D. Monds, M. W. Silby, and H. K. Mahanty, manuscript submitted) indicate that the Pst mutations affect biofilm formation and fungal inhibition differently, it is very interesting to find that there is a commonality between these phenotypes. This represents an exciting and

novel link between a developmental process and secondary metabolism. The possibility that the Pst system represents a "switch" involved with the control of both phenotypes could be of evolutionary significance. It is likely that antifungal activity and biofilm formation evolved separately, but the fact that they would often function in the same environment (rhizosphere/rhizoplane) could have led to the common use of a single switch (Pst). It is possible that PA147-2 uses the Pst system as an environmental sensor, allowing finely co-ordinated control over root colonisation by biofilm formation and expression of antifungal genes. Phosphate is an extremely important compound in biological systems - it is required for synthesis of membrane lipids, complex carbohydrates, nucleic acids, and also has a pivotal role in energy metabolism. The linking of biofilm formation and antifungal activity to a system that responds to phosphate limitation may indicate the relative importance controlling these phenotypes has for bacterial survival.

The speculations outlined above assume that the Pst defect in PAH26 results in repression of antifungal genes. Although some genes could be expressed differently in PAH26, those that are altered in the regulatory mutants PA138 and PA109 appear to be unaffected in PAH26. Therefore, any impact on antifungal genes is either subtle, or affects genes other than those regulated by *finR* and *finT*. Such regulatory effects could be more easily visualised using 2-D separation of proteins, which provides far greater resolution than the 1-D system employed here.

6.3.2 Pho regulon control by *P. aureofaciens* PA147-2.

In addition to the discovery that the Pst system is important for antifungal activity and biofilm formation, novel observations were made with respect to fundamental aspects of Pho regulon control and Pi assimilation by *P. aureofaciens* PA147-2. In contrast to reports on *E. coli* and *P. aeruginosa*, although defective in their ability to repress the Pho regulon, neither *pst* mutant exhibited constitutive Pho regulon expression. As Pi concentration was increased in the culture media, APase levels were reduced. It is important to note that even with 25mM Pi, the APase levels from PAH26 and PAB108 cultures were not as low as from cultures of PA147-2. Further to this, the mutation in *pstC* appears to have a more profound effect than the *pstA* mutation, since PAB108 consistently expressed the Pho

regulon at a higher level than PAH26. Two possible explanations for these observations are presented below.

Remaining Pst components mediate partial Pho regulon repression in PAB108 and PAH26.

Experiments with *E. coli* and *P. aeruginosa* show that, under some circumstances, components of the Pst system can compensate for the loss of other components. In *E. coli*, introduction of a nonsense codon near the start of *pstA* only resulted in a 10% derepression of the Pho regulon. This result was interpreted as showing that PstC could substitute for PstA in the formation of a partially functional repression complex (Cox et al., 1988). In *P. aeruginosa*, the loss of either PstA or PstC has no effect on repression of the Pho regulon, but the loss of both PstA and PstC completely abolishes repression (Nikata et al., 1996). These observations in other organisms point to the possibility that the components of the Pst system whose expression has not been disrupted by the transposon insertions in PAB108 and PAH26 are capable of mediating some degree of Pho regulon repression at high Pi concentrations, without the requirement for a completely intact Pst system. However, the types of mutants characterised in the studies of Cox et al (1988) and Nikata et al (1996) are fundamentally different to the Tn*5* mutants of PA147-2 in that they used substitutions and short deletions to inactivate protein products of the genes in question without causing polar effects.

It is likely that transposon insertions in *pstC* and *pstA* would have polar effects on the expression of downstream genes, suggesting that PAB108 still encodes a functional PstS, while PAH26 can express PstS and PstC. Neither strain should be able to express PstB or PhoU. Therefore, the repression of the Pho regulon by PAB108 would be solely due to PstS, while Pho regulon repression by PAH26 could be attributed to PstS and PstC. This is unprecedented, and it is generally accepted that a membrane integrated Pst complex (including PhoU) is absolutely required for proper interaction with PhoR and formation of the repression complex (Wanner, 1995). Although preliminary sequence data indicates that the *pstSCA* genes of PA147-2 are present in the same order as in other organisms, no information is available regarding whether these genes are expressed as an operon in PA147-2. Thus, although unlikely, the possibility that downstream components of the Pst

system are being expressed in PAB108 and PAH26 cannot be ruled out. If the transposon insertions in PAB108 and PAH26 only affect *pstC* and *pstA* respectively, partially functional repression complexes could be assembled in which PstA substitutes for PstC, and vice versa. In this scenario, the difference in Pho regulon repression between PAB108 and PAH26 could be explained by the different abilities of PstA and PstC to functionally substitute for one another. To examine this model it will be necessary to establish whether the *pst* genes are expressed as an operon in PA147-2, and use a technique such as RT-PCR to examine which genes are still expressed in PAB108 and PAH26.

A second system can control the Pho regulon.

The general mode of Pho regulon activation/repression by the Pst system is characterised by an "all or nothing" appearance – in response to a set threshold of external Pi, the Pho regulon is either switched on or off (Wanner, 1996). In contrast, the *pst* mutants described here exhibit a slow decrease in Pho regulon activity concomitant with increasing Pi. This suggests the possibility that some other mechanism in addition to Pst exists that can control the Pho regulon, but its effects are only observable in *pst* defective mutants. It is tempting to speculate that such a system is operating, but without considering some role for PstC, a second system lacks full explanatory power. This is because of the difference between PAB108 and PAH26, which appears to have greater control of the Pho regulon. If a second system is operating, there must still be a role for PstC, since expression of PstC is the only known difference between the two *pst* mutants. Control of the Pho regulon by systems other than the Pst complex is not unheard of. In a study of *Bacillus subtilis*, the Pst system was shown to be responsible for high affinity Pi uptake, and part of the Pho regulon, but did not appear to have a role in control of the Pho regulon (Qi et al., 1997). However, the nature of the Pho regulon control system was not described in that study.

A number of experiments need to be conducted to determine whether there is reason to implicate a second regulatory system. Of paramount importance is the examination of whether the disrupted Pst systems of PAH26 and PAB108 are sufficient to account for observations, which would allow a distinction between the propositions above. Firstly, a deletion of the entire *pst* operon in PA147-2 would be useful. If this strain showed a Pho regulon repression phenotype similar to that of PAB108, then it would be suggestive of the

involvement of another system. However, if the *pst* deletion strain was constitutive for Pho regulon expression, this would be an indication that PstS (in PAB108 and PAH26) and PstC (in PAH26) were sufficient to explain the observations. Furthermore, the phenotype of the *pst* deletion strain could be made to mimic PAB108 or PAH26 by simply expressing (*in trans*) PstS, or PstS and PstC respectively, and the relative contribution of *pstS* and *pstC* could be tested by expressing both separately and examining the impact on Pho regulon expression.

Pho regulon control in P. aeruginosa.

Although partial repression of the Pho regulon has not previously been reported in studies of *pst* mutations in *E. coli* or *P. aeruginosa*, the review of published data from a study of *P. aeruginosa* hints at such a phenomenon. In their study of *pst* genes in *P. aeruginosa*, Nikata et al (1996) constructed a *pst* deletion that removed *pstCAB* and *phoU*. This strain (NT1) may be analogous to PAB108 in that, of the *pst* genes, it can only express *pstS*. The miniTn*5* insertion in PAB108 is predicted to abolish expression of all *pst* genes except *pstS*. NT1 was reported to be constitutive for Pho regulon expression, but examination of the published data suggests that there is a 38% decrease in APase expression by NT1 after growth in 5mM Pi, relative to that after growth in a medium with no added Pi. This is in agreement with the findings of the present study, in which control experiments with PA01 and NT1 showed that the APase expression by NT1 was reduced by greater than 50% when grown in 25mM Pi, relative to APase expression in 1mM Pi. Thus, either PstS can mediate some degree of repression in *P. aeruginosa*, or there is a second system that can partially repress the Pho regulon of *P. aeruginosa* in response to increasing Pi. Like the situation with PA147-2, a mutant that lacks *pstS* as well as the rest of the *pst* genes (and *phoU*) would be required to discern between the possibilities. In contrast, a transposon insertion in *pstC* in *E. coli* led to constitutive expression of the Pho regulon in Pi concentrations up to 10mM (Daigle et al., 1995). It is thus possible to speculate that *P. aureofaciens* and *P. aeruginosa* have Pho regulon control mechanisms distinct from those of *E. coli*.

6.3.3 High affinity phosphate uptake by *P. aureofaciens* PA147-2.

Since PAH26 and PAB108 have transposon insertions in *pstA* and *pstC* respectively, it was predicted that both mutants would be defective for high affinity uptake of inorganic

phosphate. Comparisons of Pi uptake by mutants and the wildtype grown under Pi limiting and sufficient conditions were carried out, and revealed somewhat surprising results.

Pi assimilation under phosphate sufficient and limiting conditions.

As predicted, PA147-2 was unable to take up significant amounts of Pi when grown under Pi sufficient conditions. This is probably due to repression of the Pho regulon, and therefore very low expression of the Pst system. However, contrary to expectations, both PAB108 and PAH26 showed significant uptake of Pi, with PAB108 importing almost twice the amount of PAH26. The *pst* mutants should be able to uptake an equal amount or less than the wildtype, since the wildtype would have at least some (although not many) functional Pi transporters, whereas the mutants should have none. However surprising this result was, it did not indicate whether PAH26 and PAB108 had any defect in Pi transport relative to the maximum uptake by PA147-2 when grown under Pi limited conditions.

When assayed in Pi limiting conditions, PA147-2 imported the most Pi of any strain tested. PAB108 was able to import the same amount as it could when grown in Pi sufficient conditions, but this was less than 50% of that imported by PA147-2. PAH26 could uptake only 30% as much as PA147-2. These data indicate that when the wildtype and mutants are in a similar physiological condition with respect to Pho regulon activity, the mutants show defective high affinity Pi assimilation. However, this defect is not as severe as that shown by the control strain *P. aeruginosa* NT1. NT1 imported negligible Pi – the amount was comparable to that imported by PA147-2 under Pi sufficient conditions. Thus it appears that while PAH26 and PAB108 have defects in high affinity Pi uptake, the defect is quite dissimilar to that of other Pst systems – they are still capable of some degree of high affinity Pi uptake. Generally, when the Pho regulon is repressed, Pi uptake can be assumed to be the result of a basal level of Pst systems, along with low affinity uptake systems such as Pit in *E. coli* (Wanner, 1996). However, since both mutants uptake considerably more than a Pho regulon repressed wildtype, it seems unlikely that the presence of a constitutive low affinity system is able to explain the observations.

Correlation of Pi uptake capacity with Pho regulon activity.

The mutations in PAH26 and PAB108 result in different abilities to repress the Pho regulon. Interestingly, Pi uptake after five hours growth in K10P-0.1 (Pi sufficient) showed that PAB108 had a greater capacity for Pi uptake than PAH26, and this difference correlated with the different Pho regulon activity of the two mutant strains. This led to the proposal that the difference in Pi uptake is not due to different functionality of the Pi assimilation systems of the mutants, but that the Pi uptake system in both mutants is under Pho regulon control. This would explain the observation that PAB108 assimilated more Pi in K10P-0.1 (Figure 6.11), but when Pho regulon levels were more similar ($T_{0.05}$ experiments, Figure 6.10b), PAH26 and PAB108 assimilated similar amounts of Pi (Figure 6.11). Given that PAH26 and PAB108 showed a lowering of Pho regulon activity as Pi concentration was increased, if the Pi uptake system in these mutants was Pho regulon controlled it was predicted that Pi uptake would be reduced in high Pi growth conditions. The experiments on Pi uptake in K10P-5 and K10P-25 are in agreement with this prediction. They show that the more Pi in the growth medium, the lower the uptake capacity, and this correlates with Pho regulon activity results. Furthermore, the difference in reduction in Pi uptake between the mutants correlates with the different Pho regulon activities of the two strains. PAB108 is less able to repress Pho regulon activity, and correspondingly shows a lesser reduction in Pi uptake. This correlation between Pho regulon activity and Pi uptake provides persuasive support for the proposition that the mechanism responsible for Pi uptake in PAH26 and PAB108 is expressed as part of the Pho regulon in response to extracellular Pi.

6.3.4 Explaining inorganic phosphate uptake by *P. aureofaciens*.

The ability of the Pst system to import Pi is not necessarily linked to its ability to repress the Pho regulon, since the loss of one function does not always result in the loss of the other (Cox et al., 1988; Nikata et al., 1996). Thus, the mechanism by which the *pst* mutations affect Pi uptake does not have to be the same as the mechanism for loss of Pho regulon control. However, the nature of the mutations in PAH26 and PAB108 probably prevents the production of much of the Pst complex, suggesting that in this case the most parsimonious explanation for defective Pi uptake is similar to that for loss of Pho regulon control - the inability to form a fully functional Pst complex. Although this explanation is

useful to describe the defects seen in Pi limiting conditions, the effects seen at higher Pi concentrations require further examination. As has been done above for Pho regulon control, two alternative models are outlined below which could explain the observations regarding Pi uptake by *P. aureofaciens*.

A second Pho regulon controlled Pi uptake system.

The possibility that PA147-2 possesses a second Pi uptake system, in addition to Pst, cannot be excluded. To be consistent with observations, this second system must be under Pho regulon control. The phenotypes of PAH26 and PAB108 with respect to Pi uptake could be explained by inactivation of Pst by the transposon insertions, and the presence of the second system. Under high Pi, both mutants express the Pho regulon, and thus the second system imports Pi. The level of import is correlated with the Pho regulon activity, since expression (and therefore capacity) of the second uptake system would reduce with reduction of Pho regulon expression. The observation that PA147-2 takes up considerably more Pi than the mutants under Pi limiting conditions could be explained by cooperative assimilation by the two systems. The nature of any such second system is highly speculative. If it were the result of duplication of *pst* genes, the defects in Pho regulon control observed in both mutants could only be explained if the duplicated system had diverged, and lost its regulatory function. The possibility of a second Pi uptake system has some support in the literature. *Rhizobium tropici* has two Pi uptake systems that resemble ABC transporters, which are subject to induction by growth in Pi limited environments and have different affinities for Pi (Botero et al., 2000). Studies of *Mycobacterium tuberculosis* indicate the presence of multiple copies of *pstS, pstA,* and *pstC*, although only a single *pstB* has been found thus far. It is suggested that there may be several related, functional phosphate permeases (Lefevre et al., 1997). In *E. coli*, Pi can be imported by the constitutively expressed low affinity Pit system (Wanner, 1996), and the presence of a second system in *P. aeruginosa* PA01 is indicated by the biphasic Pi assimilation kinetics (Nikata et al., 1996). Any second system in PA147-2 is clearly different from those that are well characterised (*E. coli* and *P. aeruginosa*). Published data (and data presented here) show that the *P. aeruginosa* strain NT1 cannot take up an appreciable amount of Pi under limiting conditions (compared to PAB108 and PAH26). Furthermore, the Pit system in *E.*

coli is constitutive, which would be inconsistent with the data presented here that suggests Pho regulon control of Pi assimilation by Pst and the putative second system.

Partial function of Pst transporters.

An alternative to the above model is one in which the remaining components of the Pst system can form a partially and equivalently functional transporter. Based upon the operon arrangement known in other organisms, it is likely that of the *pst* genes, PAB108 can only express PstS, while PAH26 can express PstS and PstC. The condition that the transport of Pi is correlated with Pho regulon activity can be satisfied by this model because the *pst* genes are known to be part of the Pho regulon. However, it seems highly unlikely that PstS alone, or in conjunction with PstC, could form any kind of functional transport system. Even if PA147-2 can express each *pst* gene separately, and the insertions in *pstC* and *pstA* only affect those genes, the likelihood of forming a functional Pi transporter is low. Although there is a high degree of similarity between PstC and PstA, and they are both transmembrane components of the Pst system, investigations in *E. coli* and *P. aeruginosa* show that they are both required for Pi uptake (Cox et al., 1988; Cox et al., 1989; Nikata et al., 1996).

The second model outlined above seems less likely than the first to explain the observations. These models can be distinguished by the construction of mutants with the *pst* operon completely deleted. If this abolished all high affinity Pi uptake, the existence of a second, Pst-independent system would be effectively ruled out. The role for PstS and PstC could then be confirmed by expressing them separately and together in the *pst* deletion background, and monitoring the effect on Pi uptake. One problem with this approach is that if the second system was dependent upon some Pst components, its ability to import Pi would be eliminated by the *pst* deletion strain, leading to the erroneous conclusion that there is no second uptake system. Conversely, if there was only a minimal impact of Pi uptake, this would lend support to the existence of a second functional system.

Clearly, deciphering the mechanisms of Pho regulon control and Pi uptake in PA147-2 will be interesting and challenging. The fact that the Pst system seems linked to fungal

inhibition and biofilm formation are novel findings, and add to the range of complex traits whose expression appears to involve sensing inorganic phosphate concentration (Daigle et al., 1995; Gonin et al., 2000; Lucas et al., 2000). Uncovering the mechanism by which Pst influences antifungal compound production would be a great step forward in the understanding of fungal inhibition by PA147-2. A number of experiments outlined herein would be of value in the further study of this question, and the models developed provide useful start points.

Chapter 7

Field trial

7.1 Introduction.

Biological control agents are becoming increasingly popular as alternatives to chemical pesticides. Extensive laboratory studies, glasshouse trials, and limited field trials are essential before large-scale commercial development can be considered. One of the most important questions that can be addressed with field studies is, does *in vitro* antibiosis correlate with *in situ* disease suppression? Doubts surrounding this issue include the possibility that antibiotics are unstable in soil (reviewed in Gottlieb, 1976), whether antibiotics are produced in sufficient quantities to be effective *in situ* (Williams & Vickers, 1986), and whether the introduced organism can establish and survive long enough to be effective. Successful glasshouse and field studies indirectly answer these questions, and are reviewed in chapter one. An additional problem relates to the soil type. *P. fluorescens* CHAO was shown to be effective in the suppression of black rot of tobacco grown in soil containing vermiculitic clay minerals, but it was ineffective in soils with illitic clay minerals (Stutz et al., 1989). A study by Thomashow et al (1990) is a good example of a more direct approach to the question of *in situ* antibiotic synthesis and relevance. Studies on *P. fluorescens* 2-79 and *P. aureofaciens* 30-84 demonstrated that both strains produce phenazine-1-carboxylic acid (PCA) in the rhizosphere of wheat, under both growth chamber and field conditions. Furthermore, PCA production in a growth chamber correlated with suppression of take-all of wheat (Thomashow et al., 1990). Further examples are examined in chapter one, and although encouraging, it must be noted that investigations using one biocontrol organism are not directly applicable to another because of the numerous potential differences between the organisms involved.

In a previous study under glasshouse conditions (Carruthers et al., 1995), some of the issues regarding *in situ* biocontrol efficacy were examined. However, the environmentally controlled conditions of a glasshouse are quite different from the variable conditions that can be encountered in an asparagus field. Therefore it was decided that a small-scale field trial would be of value in further establishing the potential of PA147-2 as a biocontrol

agent. Since PA147-2 shows strong *in vitro* inhibition of *P. megasperma* var. *sojae*, and the previous glasshouse trials had used *Phytophthora* rot of asparagus as a model disease system, it was logical to examine the efficacy of PA147-2 in the same system under field conditions.

In this chapter, the results of a field trial in which PA147-2 was assessed for its ability to suppress *Phytophthora* rot of asparagus are presented. Since the previous glasshouse trial of PA147-2 (Carruthers et al., 1995) has been described in chapter 1, only a brief mention will be given to it in the introduction to this chapter. The results of this trial have been published (Godfrey et al., 2000, see appendix 5), and the published work forms the basis of the results shown here.

7.1.1 Cultivation of asparagus.

Asparagus (*Asparagus officinalis*) is a perennial plant with an average harvest life in New Zealand of 12 years (Robb, 1984). There are three main ways to establish asparagus. The traditional method is to transplant 10 month – 1 year old crowns from a nursery block to a field for production. Crowns are dug from the nursery during dormancy (winter), and either held in storage or transplanted immediately. A second method is direct seeding, and the third method involves raising seedlings for 10-12 weeks in a glasshouse and transplanting to fields during spring. For the first 2-3 years of its production life, asparagus is not harvested. During this time, the fern-like foliage is allowed to grow over the summer, throughout which photosynthesis leads to the production of carbohydrates that are stored in buds and storage roots attached to the crown. Fern is removed before winter, and the plant survives the winter and begins growth in spring by utilising the stored carbohydrates. Asparagus spears are harvested by cutting the stems while the buds at the tip of the stem are still tightly compressed, usually before the stems are 25cm tall.

Crop yields tend to be determined by the amount of fern foliage produced in the first three years before harvesting (P. Falloon, Pers. Comm). Thus, the health of the young plants is of paramount importance. Factors that influence the crop yields include rainfall, temperature, wind, weed competition, pests and disease. A number of diseases affect asparagus in New Zealand, including viral diseases (e.g. asparagus virus II), and soil-borne

fungal pathogens such as *Fusarium* rot and *Phytophthora* rot. *Phytophthora megasperma* var. *sojae* is a particular problem because disease resistant cultivars have not been forthcoming (P. Falloon, Pers. Comm.).

7.1.2 Phytophthora rot of asparagus

Phytophthora rot is associated with heavy rainfall (Falloon et al., 1986) and temperatures around 15°C (Falloon, 1991). In addition, young asparagus plants appear to be more susceptible than older, mature plants (Falloon, 1991). Thus, *Phytophthora* rot can be a problem with long lasting consequences, given the importance of vigour in young asparagus ferns. *Phytophthora* rot can lead to significantly reduced plant emergence (Falloon et al., 1991), and it has been suggested that two consecutive years of disease could result in a permanent decrease in productivity of the affected plants (Falloon et al., 1986).

Dispersal of *Phytophthora* is dependent upon water, which explains the increased severity of outbreaks in wet conditions. *Phytophthora* zoospores move passively in water currents, but are also motile and tactic toward growing root tips and wounds (reviewed in Carlile, 1983). Presumably the taxis is toward exudates from growing or wounded roots. Within 30-60 minutes of zoospore entrapment, cysts germinate and form germ tubes that penetrate asparagus tissue. The subsequent development of active mycelia results in the rotting of feeder roots, storage roots, crowns, spears and fern stalks. This process leads to the observable symptoms described below.

SYMPTOMS.

The most revealing symptoms of disease occur below the ground, on the roots. While healthy roots are firm, opaque, white, and have numerous feeder roots, *Phytophthora* rot infection results in pulpous, cream-coloured storage roots that appear partially transparent and water-soaked (Carruthers et al., 1995) (Figure 7.1). As the disease progresses, feeder roots become water soaked and after several weeks of infection are often completely absent.

Figure 7.1. Asparagus crowns with and without *Phytophthora* rot. a. Healthy asparagus crown. b. Diseased asparagus crown showing the water-soaked appearance of the roots typically associated with *Phytophthora rot.*

Above the ground, symptoms begin with wilting of fern fronds, foliage browning, and eventual death (Figure 7.2). Although the disease requires water to begin, it may be during water stress that the symptoms become visible above the ground. This is due to the damage to the roots, which leaves the plant unable to meet the water demands of the foliage (P. Falloon, Pers. Comm.).

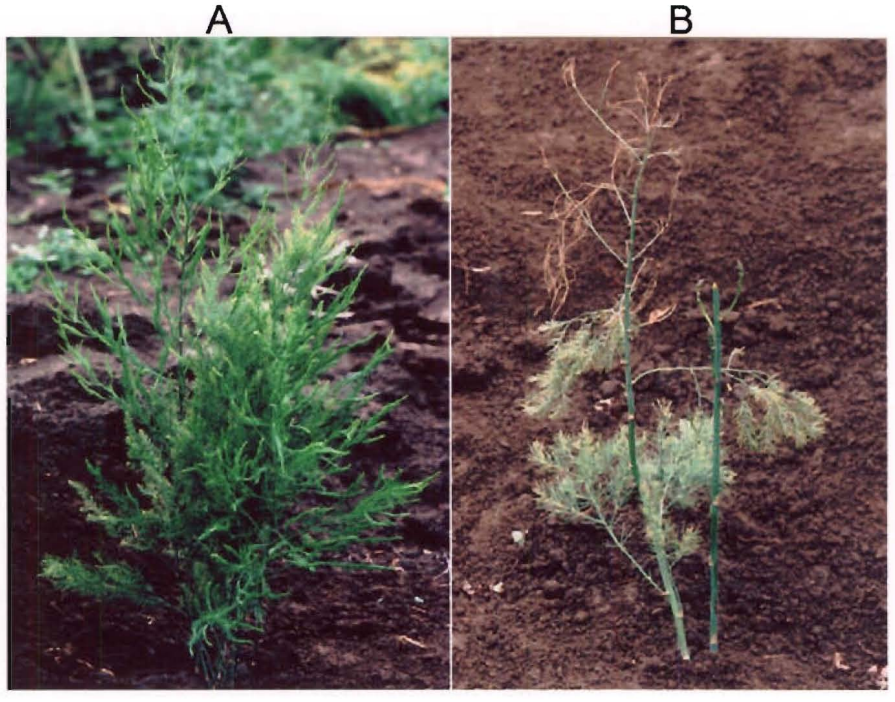


Figure 7.2. Asparagus ferns with and without symptoms of *Phytophthora* rot. **a.** Healthy asparagus fern. **b.** Asparagus fern showing symptoms of *Phytophthora* rot. Note the reduced overall vigour, and the browning of the extremities.

CHEMICAL CONTROL.

In New Zealand, *Phytophthora* rot is widely controlled by using formulations that contain metalaxyl (P. Falloon, Pers. Comm.), which acts by inhibition of fungal RNA synthesis (Davidse, 1984). Use of metalaxyl is recommended during transplantation of crowns, at which time crowns should be dipped. In addition, metalaxyl can be applied to a field of established asparagus 10-14 days before harvest. Metalaxyl is more effective as flooding decreases (Falloon et al., 1991), indicating that in extremely wet seasons, metalaxyl may not be completely effective against *Phytophthora* rot. Thus, the problem of *Phytophthora* rot has not been completely solved by the use of metalaxyl, and alternatives such as biocontrol are worthy of investigation.

In a glasshouse study, Carruthers et al (1995) examined the significance of antibiotic production by PA147-2 for the control of *Phytophthora* rot. Using PA147-2 and an antibiotic defective mutant, Carruthers demonstrated that disease suppression was correlated with the ability to produce antifungal compounds. Treatment with PA147-2 reduced disease from 97% (control) to 72%, while treatment with the antifungal mutant PA109 had no impact on disease occurrence. While a number of plants treated with PA147-2 still showed disease, the severity was reduced.

The observation that PA147-2 reduced disease severity on asparagus was important given that it is plant vigour in the first few years of life that determine long term productivity. The ability to reduce disease severity would be valuable to growers, especially organic growers who do not use chemical treatments. To further examine the potential of PA147-2, the field trial objectives below were proposed.

7.1.3 Field trial objectives.

1. The primary objective of the field trial was to examine the performance of PA147-2 in the suppression of disease under field conditions. Because the antifungal mutants are considered to be genetically modified organisms, it was not possible to include one in the trial as a control. Therefore, this objective was not intended to explicitly examine the role of antibiosis in disease suppression. Instead, it was decided to assess the performance of PA147-2 relative to Ridomil® 250EC, a metalaxyl-based fungicide.

2. In addition to disease suppression, the survival of PA147-2 in the field was of interest. Thus, the second objective was to monitor the population of PA147-2 in the soil and rhizosphere.

7.2 Experimental design.

7.2.1 The field trial.

The trial was carried out over six months (September 1996 – March 1997) in an isolated area of Eyre shallow fine sandy loam soil inoculated with *P. megasperma* var. *sojae* using 22-month-old asparagus crowns (cultivar UC157 F1 hybrid) previously grown in Templeton silt loam soil. Planting of treated crowns was performed using a randomised complete block design (Steel & Torrie, 1976). Each plot consisted of 20 crowns planted 300 mm apart within a single row at a depth of 80 mm. Each treatment was replicated 4 times. Dams (200 mm high) were erected at the end of each plot to prevent leaching and cross-contamination of treatments. Guard rows were planted on the sides of the trial. Randomly selected crowns in the guard rows were inoculated with PA147-2 for use in the population study experiment. General maintenance of plants consisted of 25 mm of irrigation every 14 days applied over a 3-h period. Because saturated or near saturated soil conditions are essential for *Phytophthora* zoospore production and dispersal (Falloon & Tate, 1986), flooding of the plot was achieved by irrigating for extended time periods (Table 7.1). Fertiliser, insecticides, and herbicides were applied as indicated in Table 7.2.

Table 7.1. Watering schedule applied to asparagus (*Asparagus officinalis*) field trial plot.

Date (1996)	**Irrigation (mm)**	**Time Period (h)**
26 Sep	100	8
27 Sep	200	16
17 Oct	200	16
18 Oct	200	16
24 Oct	100	8
25 Oct	200	16

Table 7.2. Chemicals applied to asparagus (*Asparagus officinalis*) field trial plot

Date	Chemical applied	**Description**	*Amount applied* **(kg ha^{-1})**
2 Oct 1996	Karmex	Herbicide	2
11 Dec 1996	Malathion	Insecticide	2
13 Dec 1996	Linuron	Herbicide	1
14 Dec 1996	Crop Master 13	Fertiliser	350
18 Jan 1997	Malathion	Insecticide	2
2 Feb 1997	Malathion	Insecticide	2
16 Feb 1997	Malathion	Insecticide	2

Harvest was carried out 160 days after planting. The fern from each plot was cut at soil level, washed with tap water to remove soil particles and placed into paper bags. Following oven drying at 75°C for 72 h, dry weights of the fern were determined.

7.2.2 Experimental treatments.

Four experimental treatments were used. Treatment 1 was the application of a commercially available fungicide, Ridomil® 250EC, which is applied for the control of *Phytophthora* rot of asparagus throughout New Zealand. In Treatment 2, asparagus crowns

were left untreated. Treatment 3 was the inoculation of asparagus crowns with PA147-2. The fourth treatment was the application of LB medium to crowns. The four treatments allowed the comparison of PA147-2 treatment to a current chemical control, and to asparagus that has no "added protection". In addition, any unexpected effect of the bacterial growth medium could be assessed.

7.3 Materials and Methods.

Materials and methods other than the design of the experiment are described in chapter 2.

7.4 Results.

7.4.1 Disease severity.

Phytophthora megasperma var. *sojae* disease symptoms were observed on asparagus plants at harvest (Figure 7.3), indicating that *Phytophthora* was well established in the plot. Symptoms ranged from reduced fern vigour with slight browning of fern tips, to stunted growth and wilting with browning over most of the plant.

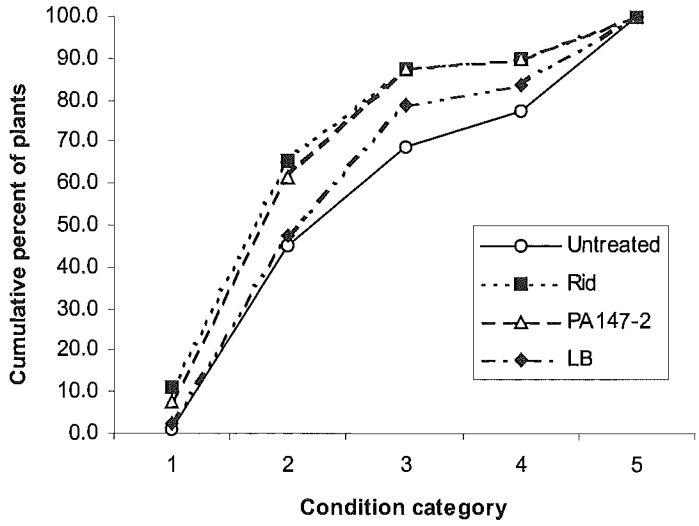


Figure 7.3. Disease severity visually observed from plants growing in diseased plot. Individual asparagus plants were visually graded on overall plant condition on a scale of 1-5 to indicate percentage of foliage affected by disease (1 = 0%, 2 = 1-20 %, 3 = 21–40%, 4 = 41-80%, 5 = 81–100%).

When visual disease severity data were analysed using an ordinal logistic model it was found that there was no significant heterogeneity, so treatment effects were tested against theoretical multinomial values. Fitted model coefficients for Ridomil® 250EC, PA147-2, and LB were –1.1, -0.9, and –0.2 respectively. The standard error of these coefficients was 0.30. Fern dry weights are shown in Table 7.3. Visual observation of the trial plants indicated that treatment of asparagus crowns with Ridomil® 250EC provided a high degree of protection against *Phytophthora* rot. In contrast, the untreated and LB treated controls were severely affected by *Phytophthora* rot, with all plants showing some degree of disease. The plants treated with PA147-2 showed signs of disease, although the symptoms were significantly less severe than those displayed by the untreated plants.

Table 7.3. Summary of asparagus (*Asparagus officinalis*) fern dry weights used in statistical analysis.

Treatment	**Weight (g)**
Untreated	160
Ridomil® 250EC	437
PA147-2	252
LB	186
LSD (5%), 7 d.f.*	109

* degrees of freedom

7.4.2 Effect of PA147-2 on disease severity.

PA147-2 improved dry weights of fern by almost 60% (significant at 5% level using a one-sided test), but Ridomil® 250EC improved dry weights by much more, averaging 173% more than untreated plants.

7.4.3 Bacterial population study.

UNTREATED CROWNS.

Comparison of Rf^R isolates from untreated crowns revealed four morphologically dissimilar colony groups which API 20 NE strip analysis revealed to be three *P. fluorescens* biotypes (0147475, 0147755, and 0147775) and one *P. aureofaciens* strain (0147577). Bioassay

revealed one of the three *P. fluorescens* biotypes (0147755) to have antifungal activity toward *P. megasperma* var. *sojae*.

PA147-2 TREATED CROWNS.

Culturable Rf^R bacteria (CFU g^{-1} of soil) isolated from PA147-2 inoculated crowns are presented as a logarithmic linear graph with comparison to Rf^R CFU g^{-1} of soil isolated from control crowns (Figure 7.4)

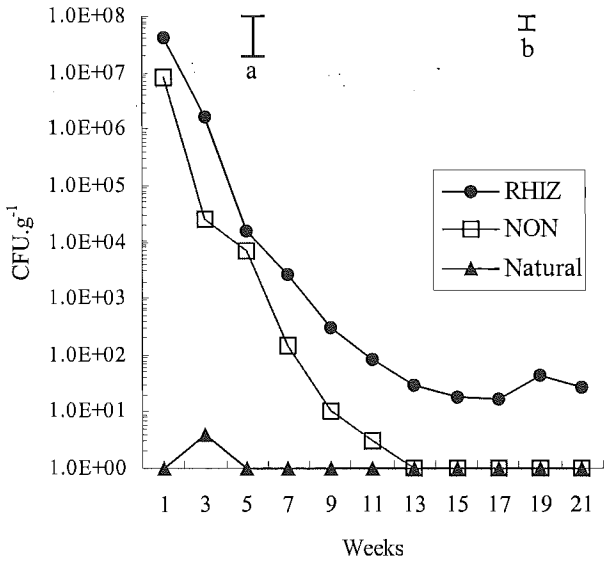


Figure 7.4. Rifampicin-resistant bacteria isolated during the field trial. (RHIZ = bacteria isolated from the rhizosphere of inoculated plants; NON = bacteria isolated from soil surrounding the roots of inoculated plants; Natural line shows the rifampicin resistant bacteria isolated from control plants that were not inoculated with PA147-2). Data presented are the mean from four samples (a = LSD (5 %) for comparing Rhiz with Non-rhiz positions at any of weeks 1-11 (d.f. =18); b = LSD (5 %) for comparing Rhiz mean with zero, for weeks 13-21 (d.f. = 15)).

Comparison of all Rf^R bacterial isolates from PA147-2 inoculated crowns indicated six morphologically distinguishable colony types. A single representative of each was purified onto PMM and labelled as PA147-2 crown isolate (PACI) A - F. When PACI isolates A-F were bioassayed against *P. megasperma* var. *sojae*, all demonstrated antifungal activity

comparable to PA147-2 after seven days. PACI isolates A-F exhibited siderophore production on PMM agar after five days with equal fluorescence intensities to PA147-2. LB agar supplemented with the respective antibiotic revealed that all PACI isolates A-F had resistance to Rf and Ap but were sensitive to Km, Gm, Nal, Strep, and Tc, like PA147-2. Gram stain preparation of PACI isolates revealed Gram-negative rods, identical in size and cellular morphology to PA147-2. Biotypes obtained using API 20 NE were identical for PACI isolates and PA147-2 (0147577). 0147577 is 83% confirmation of a "very good identification to the genus *P. aureofaciens*" (1996 API Software Database). Southern hybridisation analysis showed that a 16kb *Eco*RI fragment of DNA derived from PA147-2 which is involved in antifungal activity (chapter 5) hybridised with an identically-sized *Eco*RI DNA fragment in PACI isolates, supporting the identification of the PACI isolates as PA147-2 (data not shown).

7.5 Discussion.

The field trial presented in this chapter was carried out to see whether inhibition of *Phytophthora* rot of asparagus by PA147-2 observed in glasshouse trials (Carruthers et al., 1995) could also be demonstrated under field conditions. These are preliminary findings of the field application of PA147-2 as a biocontrol agent against *P. megasperma* var. *sojae.*

7.5.1 PA147-2 compared to Ridomil® 250EC.

Fern dry weight was used as a measure of plant vigour because fern vigour of asparagus plants in the first three years of planting is proportional to the yield of spears obtainable over future (Scheer & Ellison, 1960). The percentage increase in total fern dry weight obtained from plants treated with Ridomil® 250EC and PA147-2 was calculated in comparison to the fern dry weight obtained from untreated plants. Dry weight increases relative to the untreated control were considered to result from disease suppression provided by the respective treatment. Treatment with Ridomil® 250EC and PA147-2 resulted in dry weight increases of 173 and 55.8% respectively. Although Ridomil® 250EC shows considerably greater protection than PA147-2, the bacterial treatment is still significantly better than the untreated control (5% LSD). It is interesting to note that treatment with LB resulted in a slightly greater fern weight than the untreated plants. One

can speculate that the LB may have stimulated growth of indigenous bacteria on the crowns, resulting in either greater competitive exclusion of the pathogen, or production of antifungal metabolites by the resident microbes. This effect would be short lived because the LB concentration would be reduced quickly after planting and watering.

One of the Ridomil® 250EC treated plots only provided 21% of its expected dry weight of fern, after adjusting for systematic local variation (which comprised block effects and a 30% reduction of fern dry weight in plots along one edge of the trial near some shelter trees), so data from this plot were excluded from statistical analysis of fern dry weights.

7.5.2 Survival of PA147-2 in the field

During the field trial, monitoring of PA147-2 survival was achieved by periodic re-isolation. Based on differences in colony morphology, initial doubt was raised as to whether all Rf^R bacteria isolated from PA147-2 inoculated crowns were the same *P. aureofaciens* PA147-2 strain used for inoculation. Subsequent phenotypic analysis indicated that those Rf^R isolates chosen for analysis were indistinguishable from PA147-2 and Southern hybridisation indicated genetic similarity to a 16kb genomic region from PA147-2 previously shown to be involved in antifungal activity (Carruthers et al., 1994). The result of the Southern hybridisation supports the identification of the PACI isolates as PA147-2, although 16s rDNA sequencing and macro-restriction enzyme profiling using FIGE would provide a more definitive identification. The variation in morphology might not be too surprising given that when populations of *P. fluorescens* have evolved to occupy different niches, this has been concomitant with changes in morphological characteristics (Rainey & Travisano, 1998). Although not all Rf^R bacteria isolated from PA147-2 inoculated crowns were analysed by Southern hybridisation, the number of Rf^R bacteria isolated from PA147-2 inoculated crowns far exceeded natural background levels of Rf^R bacteria therefore suggesting Rf^R bacteria isolated were an accurate estimation of PA147-2 survival (Figure 7.4).

The observation that the PA147-2 population declined over time indicates that PA147-2 might be unable to establish and maintain high populations when inoculated into new field

environments. When sampling was stopped, PA147-2 was present at approximately 100 cfu/g of soil in the rhizosphere, and was undetectable in bulk soil. Whether the rhizosphere population was stable is unclear. The low population density could reflect a lack of competitive fitness, which is a potential problem for a biocontrol agent. One possible cause of a lack of fitness is the metabolic load of producing antifungal compounds. This is addressed in a preliminary, *in vitro* study in chapter 8.

Results of this field study have indicated that the direct inoculation of asparagus crowns with PA147-2 before planting resulted in a 55.8% increase in fern yield compared to the untreated control. Disease control is essential during wet field conditions (achieved here by flooding, see Table 7.1), when *Phytophthora* zoospore production and dispersal is maximal (Falloon et al., 1985). These conditions are encountered early in the growing season, and in this study the extensive watering period correlates with the period in which PA147-2 numbers were highest (the first 2 months). The results of the bacterial population study suggest that PA147-2 survival was sufficient during the trial to enable fungal inhibition by either: 1) the production of a sufficient quantity of antifungal compounds resulting in inhibition of *P. megasperma* var. *sojae* establishment; or 2) the inhibition of the pathogen by the introduction of microbial competition due to the establishment of PA147-2 in the rhizosphere. Specific experiments could be designed to examine which of the above mechanisms is responsible for disease suppression, or whether suppression results from the combined action of antibiotic production and microbial competition. The previous glasshouse trial (Carruthers et al., 1995) provides strong support for the suggestion that disease suppression is linked to suppression of the pathogen by synthesis of antifungal compounds.

7.5.3 Prospects for using biocontrol.

The observation that PA147-2 inoculated asparagus plants showed a 55.8% increase in fern dry weight compared to untreated plants indicated the potential of PA147-2 as a biocontrol agent. However the fungicide Ridomil® 250EC, provided a 173% increase and therefore re-emphasises that biological control of *Phytophthora* rot of asparagus with *P. aureofaciens* still needs extensive investigation before becoming a viable option that provides comparable protection to that given presently by chemical pesticides. As a result of the

increasing demand being generated in the area of organic farming (Figiel, 1994), PA147-2 application may provide an alternative non-chemical option resulting in an increase in crop yields. Application of PA147-2 at the time of planting will provide protection to the plants as they become established, but the bacterial population study suggests that the bacterial numbers will be very low by the time of harvest which is important when considering the issue of human consumption. In addition, the use of an integrated strategy of pest management in which PA147-2 is used in combination with a chemical fungicide may allow a reduction in the amount of chemicals applied to crops. Although such an approach would not eliminate the use of chemicals, the reduced need for them could have both economic and environmental benefits. Future trials would be useful for three reasons. First, further investigations involving larger trials with more sophisticated bacterial application techniques will enable a more extensive analysis of the ability of PA147-2 to inhibit *Phytophthora* rot of asparagus. Such a study would naturally build upon this initial, small-scale work, and using modern approaches to soil inoculation (e.g. pellet formulations) would improve upon the rather crude approach used in this study. Second, it would be useful to isolate the biocontrol agent from experimental plants (rather than guard row plants as in this study), in order to assess the effects of any interactions between the bacterium and the pathogen. Finally, long-term studies would allow an assessment of whether biological control of disease in the early life of asparagus leads to an increased crop yield when the plants begin to produce harvestable spears.

The potential of PA147-2 to control *Phytophthora* rot is clear, and it would be interesting to extend the investigation into protection of other crops. The conditions under which *Phytophthora* disease develops are rather harsh, and the relative success of PA147-2 under these conditions is encouraging. In crops where disease develops more readily it is possible that PA147-2 would be even more effective since the bacteria might not have to endure such stressful conditions. Also, the extension of the analysis into crops that are sown commercially from seed would allow the efficacy of application by seed coating to be examined.

7.6 Acknowledgment.

This field trial was undertaken with Scott Godfrey, who contributed equally to the work. The advice and assistance of Peter Falloon were essential to the successful execution of the field trial.

Chapter 8

In vitro competition between PA147-2 and antifungal mutants

8.1 Introduction.

Many fluorescent *Pseudomonas* spp. that inhabit soil are capable of producing antifungal metabolites. As discussed (chapter 1), the possibility that these organisms might be exploited as biological alternatives to chemical control of agricultural diseases has led to intense investigation of regulation and synthesis of compounds that inhibit fungal growth. Such investigations have yielded mutants that no longer produce antifungal compounds, and these mutants have been used to demonstrate the importance of antifungal metabolites for biocontrol of fungal diseases of crop plants (Carruthers et al., 1995; Howie & Suslow, 1991; Keel et al., 1990; Thomashow & Weller, 1988). The successful use of bacteria in biological control is dependent upon an evolutionary adaptation that presumably allows the bacterium to compete with fungi in their shared niche. However, biocontrol involves the application of these bacteria to new environments, a situation in which bacterial fitness is of critical importance. The introduced organism must be sufficiently fit to compete with the indigenous microbes and establish a population. A number of studies have demonstrated the effects of genetic modification of bacteria with respect to fitness in laboratory and soil microcosm systems. Generally these experiments have examined the impact of genetic markers, and have found both positive (Blot et al., 1994) and negative effects (Brockman et al., 1991; De Leij et al., 1998; Orvos et al., 1990) associated with modification. Such investigations aid in the choice of marker systems for monitoring organisms introduced into soil environments. Though these studies clearly demonstrate the impact on fitness of several marker systems relative to wildtype strains, they do not provide insight into the absolute fitness of unmarked strains.

Pseudomonas aureofaciens PA147-2 inhibits the *in vitro* growth of a number of phytopathogenic fungi (Carruthers et al., 1994), and can suppress *Phytophthora* rot of asparagus in glasshouse and field trials (Chapter 7; Carruthers et al., 1995; Godfrey et al., 2000). In chapter 7, it was shown that while PA147-2 could suppress *Phytophthora* rot in the field, this protection was only a ~60% increase in asparagus fern weight as compared to

a 173% increase provided by a chemical fungicide relative to untreated controls (see chapter 7). Furthermore, the population of PA147-2 decreased considerably over the course of the trial indicating that despite the high initial inoculation density, PA147-2 was displaced by indigenous microbial populations. This was of interest because laboratory studies have indicated that PA147-2 can efficiently colonise sterile plant roots (R. D. Monds and H. K. Mahanty, unpublished data). Two questions arising from the field study were (i). Could the lower level of disease control be related to the declining population of PA147-2; and (ii) could the apparent inability of PA147-2 to establish a high population be due to a lack of competitive fitness? A question that can be derived from these is whether producing antifungal metabolites is associated with a fitness cost. It is intuitively obvious that there would be a metabolic cost to producing antifungal compounds because the process would require energy. However, does the metabolic load translate to reduced fitness, and does any such fitness cost have the potential to result in the failure of PA147-2 as a biocontrol agent? As a preliminary means to examine these questions, *in vitro* competition experiments between PA147-2 and five Tn*5*-generated antifungal-deficient mutants have been carried out. It was reasoned that if there was a fitness cost involved in producing antifungal compounds, one or more mutant should display increased fitness and thus outcompete the wildtype under these selectively neutral conditions. However, if the inhibition of fungal growth does not represent a significant metabolic investment by PA147-2, mutants and wildtype should be similarly fit under the conditions employed.

8.1.1 Objectives.

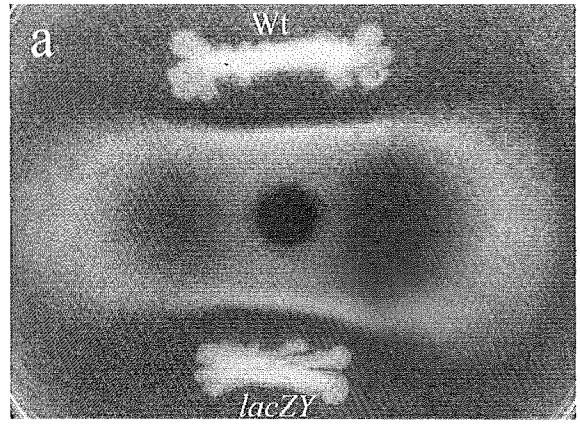
The work presented in this chapter began with a single objective, which was to determine whether PA147-2 was less fit than the antifungal mutant derivatives. Such a result would indicate that there is a fitness cost to production of antifungal metabolites by PA147-2. After the initial experiments were completed, further objectives were defined.

1. What effect, if any, does aging PA147-2 for ten to 20 days in culture have on its ability to compete?
2. Do antifungal mutants arise spontaneously during starvation?

8.2 Results.

8.2.1 Construction and tests of fitness of PA147-2*lacZY*.

Before any competition assays were possible, it was necessary to introduce a selectable marker into PA147-2, so that it could be distinguished from the Km^R Tn*5* mutants. The introduction of Tn*7*-*lac*7117 (*lacZY*) into PA147-2 resulted in the creation of PA147-*lacZY*, which allows selection based upon the ability to utilise lactose and differentiation from lac$^-$ strains when plated on media containing Xg . To ensure that the incorporation of Tn*7* and the expression of *lacZY* had no uncontrolled effects, the newly created PA147-2*lacZY* was tested for fungal inhibition, growth rate, and competition against wildtype PA147-2 (Figure 8.1). PA147-2*lacZY* was able to inhibit the *in vitro* growth of *R. solani* and *G. graminis* var. *tritici* as strongly as the wildtype (Figure 8.1a).



b.

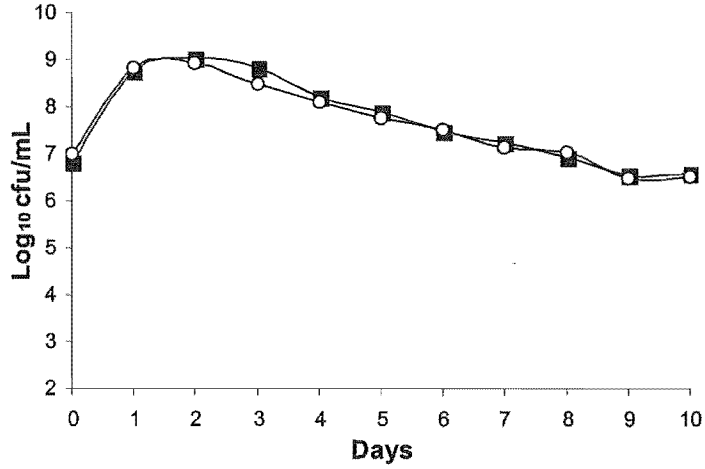


Figure 8.1. Experiments to examine the impact of Tn*7*::*lacZY* insertion on important phenotypes of *P. aureofaciens* PA147-2. **a.** *In vitro* inhibition of the plant pathogenic fungus *G. graminis* var. *tritici* by PA147-2 (Wt) and PA147-2*lacZY* (*lacZY*). **b.** Competition between PA147-2 (■) and PA147-2*lacZY* (○)

The growth rate of PA147-2*lacZY* was indistinguishable from that of PA147-2, and the competition experiment showed that PA147-2 and PA147-2*lacZY* appear to be equally fit under starvation conditions, with neither strain dominating the culture (Figure 8.1b). These results suggest that there was no fitness cost to expression of *lacZY* under conditions relevant to this study.

8.2.2 Competition between PA147-2*lacZY* and Tn*5* mutants (1:1 ratio).

Five Tn*5* generated antifungal mutants were used in initial competition experiments with PA147-2*lacZY*. The results for 1:1 competitions are summarized in Figure 8.2. These data show that the mutants can be divided into two categories based upon their competitive ability relative to PA147-2*lacZY*. One group (comprising PAI95, PAH26, and PAE21), appear to be of equivalent fitness compared to PA147-2*lacZY*, while members of the second group (PA109 and PA138) both showed an ability to outcompete PA147-2*lacZY* under these conditions.

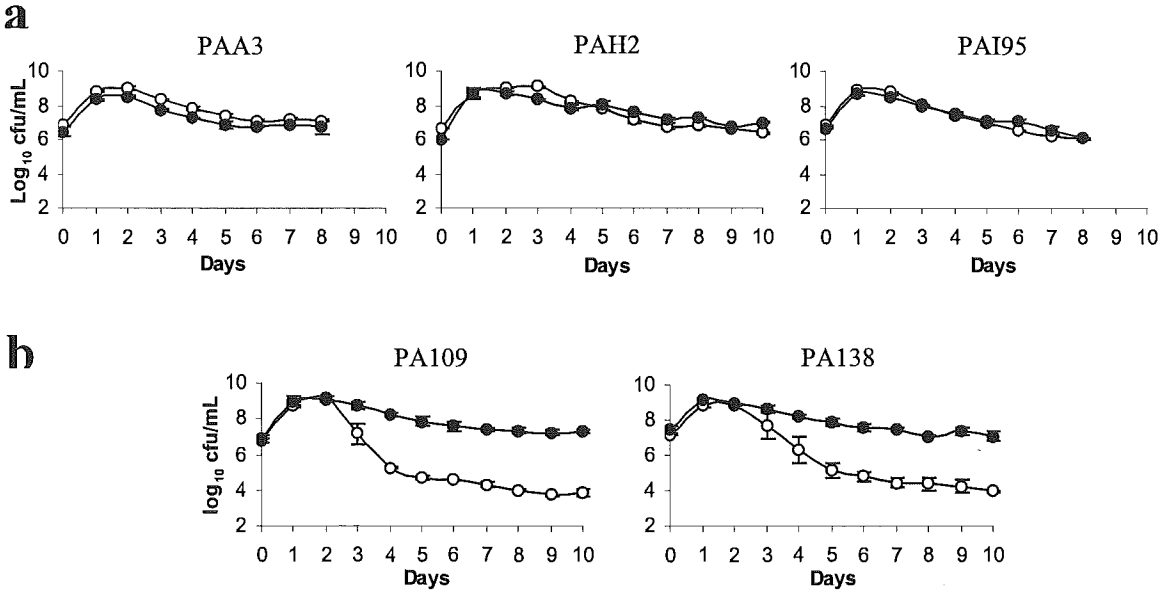


Figure 8.2. *In vitro* competition between PA147-2 and antifungal-defective mutants in Pseudomonas minimal medium. For competition experiments between PA147-2*lacZY* (○) and Tn*5*-generated antifungal minus mutants (●), approximately equal numbers of group 1 mutants (**a**) or group 2 mutants (**b**) were mixed with PA147-2 in PMM and grown for eight to ten days. Strains were enumerated daily. Data shown represent the averages from three independent experiments. Standard errors are shown, but error bars are sometimes hidden by the data point.

However, it is worth noting that in competition with PA138 and PA109, PA147-2*lacZY* was always detectable – the wildtype continued to persist as a minority population for as long as the experiments continued. Analysis of growth rate showed that all five mutants were comparable to PA147-2*lacZY*, suggesting that the initial exponential growth rate could not account for the differences observed.

8.2.3 Does the Tn*5* transpose in starved cultures?

One possible explanation for the observation that PA138 and PA109 have higher fitness than PA147-2*lacZY* is that the Tn*5* element is unstable, and transposes randomly. Such transposition might yield fitter mutants, and the fitness of these mutants would cause them to be enriched in the population. To examine this possibility, Southern analysis was used. Two monocultures of PA138 and PA109 were grown for ten days, after which total DNA was prepared and probed with a Tn*5* specific probe. If the transposon had transposed on a number of occasions, hybridisation would be seen with more than one *Eco*RI fragment, whereas if the transposon were stable, hybridisation would occur as it does in the original strains. The results (Figure 8.3) show that the DNA isolated from starvation cultures had Tn*5* in *Eco*RI fragments of the same size as the unstarved mutants.

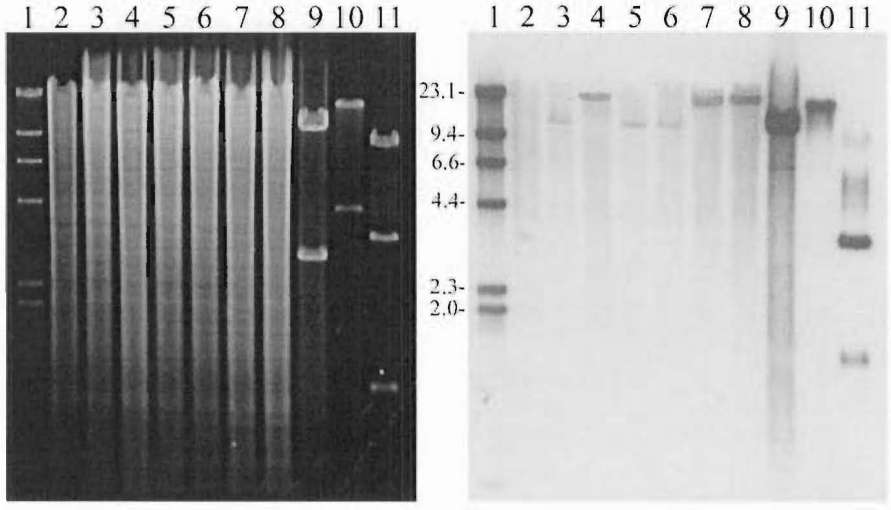


Figure 8.3. Southern analysis of Tn*5* stability in competition cultures. Total bacterial DNA was digested with *Eco*RI, and probed with a Tn*5* probe. Lanes are: 1,. Lambda *Hin*dIII standard; 2, PA147-2; 3, PA138; 4, PA109; 5 and 6, Starved PA138; 7 and 8, Starved PA109; 9, pCM138E control; 10, pFC109 control; 11, pSUP2021 digested with *Hin*dIII (source of the probe). The probe has hybridised with an 11.5kb fragment in starved and non-starved PA138, and in the pCM138E control. The probe has hybridised with an approximately 21kb fragment in starved and non-starved PA109, and in the pFC109 control.

8.2.4 PA138 as a minority inoculum.

To further examine the competitive nature of the group 2 mutants relative to PA147-2*lacZY*, competition experiments were conducted in which the ratio of PA138 to wildtype inoculum was 1:250. In these experiments, PA138 was able to establish and eventually dominate the cultures (Figure 8.4a). This is in contrast to control experiments in which PA147-2*lacZY* was inoculated as an average 1:125 minority population in competition with PA147-2. In this control, the minority wildtype was unable to establish a high population, and the ratio of strains remained comparable over the ten days (Figure 8.4b).

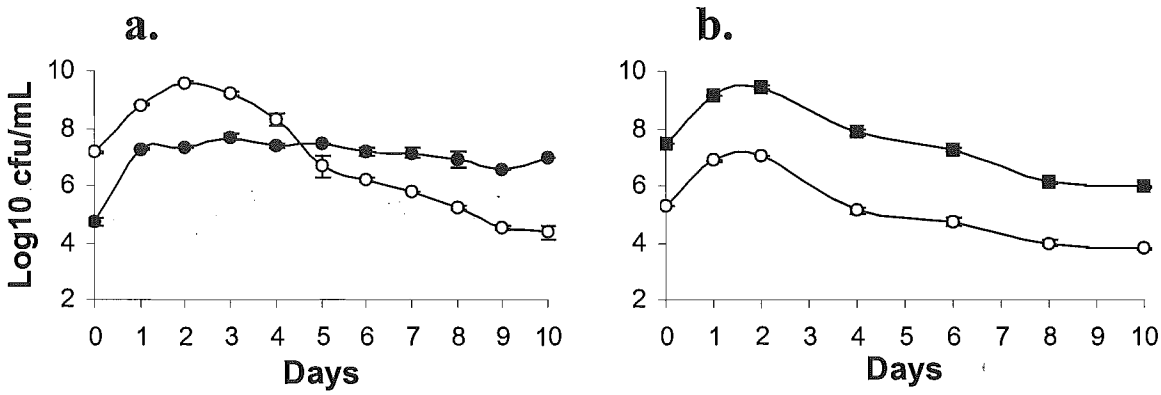


Figure 8.4. Competition experiments involving strains inoculated as minority populations. **a.** Competition between PA138 (●) and PA147-2*lacZY* (○). PA138 was inoculated as a 1:200 minority population relative to PA147-2*lacZY*. Despite the low initial inoculum, PA138 was able to outcompete PA147-2*lacZY*. **b.** Control experiment. PA147-2*lacZY* (○) was inoculated as a 1:125 minority relative to PA147-2 (■). PA147-2*lacZY* was not able to increase its relative representation in the population.

8.2.5 Determination of DNA sequence flanking Tn*5* insertions.

DNA sequences flanking the transposon insertion points are described in chapter 4. Of particular interest is the finding that the transposon in PA138 had interrupted a sequence whose predicted translation product resembles members of the LysR family of transcriptional regulators, and the insertion in PA109 interrupted a putative two-component regulator gene. The group two mutants appear to have mutations in global regulator genes, which are likely to lead to altered expression of large numbers of genes (see chapter 5). This is in contrast to the group 1 mutants PAH26 and PAI95 whose mutations would be expected to have more specific effects, since they do not appear to disrupt genes encoding regulatory proteins. Since the other group 1 mutant (PAA3) has an insertion in an

apparently novel sequence it is not possible to predict the global impact of the mutation, beyond the fact it does not lead to increased fitness.

8.2.6 Do antifungal mutants arise in PA147-2 monoculture?

The ability of PA138 and PA109 to outcompete PA147-2 bears a striking resemblance to the so-called GASP phenomenon, which is well studied in *E. coli* (Kolter et al., 1993; Zambrano & Kolter, 1996). During prolonged starvation, the GASP (growth advantage in stationary phase) mutants proliferate and take over cultures, displacing the less fit parental strain. Such take-overs occur more quickly than population shifts in continually growing cultures, reflecting the versatility of the bacterial response to starvation (Huisman et al., 1996; Zambrano & Kolter, 1996). This phenomenon has been associated with a number of different mutations, notably in *rpoS* (Zambrano et al., 1993) and *lrp* (Zinser & Kolter, 2000). RpoS is a sigma factor involved in the regulation of a cascade of genes in response to the transition to stationary phase growth (Lange & Hengge-Aronis, 1991), and Lrp (the leucine-responsive regulatory protein) is a transcriptional regulator of an extensive regulon comprised of between 35 and 75 genes in *E. coli* (Newman et al., 1996). Our group 2 mutants show similar abilities to rapidly outcompete their parental strain, and both appear to have insertions in genes that encode global regulators. The similarity between group 2 mutants and the GASP phenotype led to the speculation that the group 2 mutants were analogous to GASP mutants. It was predicted that mutants of higher fitness than the wildtype would arise during prolonged starvation of PA147-2, and that these mutants would dominate the culture. Since PA109 and PA138 are also antifungal minus, it was of interest to address the possibility that some of the fit mutants would also be antifungal mutants. This is of particular interest from a biocontrol perspective. To address the possibility that antifungal mutants arise in PA147-2 monoculture, two independent cultures of PA147-2 were grown in PMM for 20 days. Individual colonies from both cultures were tested for their ability to inhibit fungal growth. The results (Table 8.1) show that antifungal minus and reduced mutants arise in PA147-2 monocultures, but there is extreme variation between replicates. Previous long term starvation experiments have also shown considerable variation between initially identical lines (Finkel & Kolter, 1999).

Table 8.1. Fungal inhibition by colonies arising in starvation monoculture[a]

Replicate	Antifungal plus	Antifungal reduced	Antifungal minus
Number one	80/157	4/157	73/157
Number two	72/95	20/95	3/95

[a] Two cultures of PA147-2 were grown for 20 days in Pseudomonas minimal medium. Upon plating, colonies from both cultures were assessed for ability to inhibit fungal growth. Data are expressed as number of colonies with a particular phenotype over total colonies tested.

8.2.7 PA138 and PA109 vs aged PA147-2*lacZY*.

If the observations outlined above are a consequence of GASP evolution it follows that aged cultures of PA147-2 (and PA147-2*lacZY*) will have accumulated mutants of greater fitness *in vitro* than the original ancestral strain. A prediction of this hypothesis is that PA109 and PA138 would be less able to displace populations derived from aged cultures than 'young' PA147-2 cultures in competition experiments. To test the hypothesis, competition experiments were inoculated with 'young' PA109 (or PA138) and either ten or 20 day old PA147-2*lacZY*. Consistent with predictions, neither PA138 nor PA109 were as competitive against the 'aged' PA147-2*lacZY* (Figure 8.5). In competition against 'young' PA147-2*lacZY*, PA109 and PA138 eventually establish a population up to four orders of magnitude higher than that of PA147-2*lacZY*. However, in these competitions against 'old' PA147-2*lacZY*, the difference was never more than one order of magnitude.

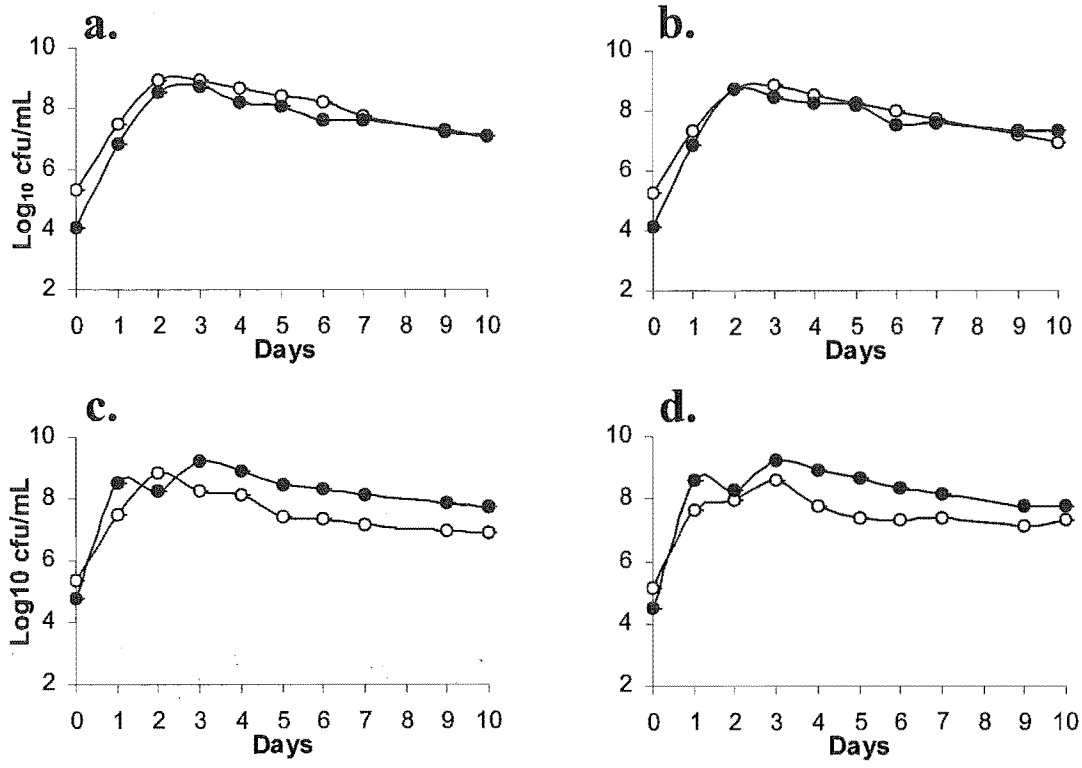


Figure 8.5. Competition between group 2 mutants and aged PA147-*2lacZY*. Competition between PA138 (a, b;●) or PA109 (c,d; ●) and either 10 day old (a,c) or 20 day old (b,d) PA147-*2lacZY* (○). The aged PA147-*2lacZY* is fitter than the parental PA147-*2lacZY*, as evidenced by the increase in its competitive fitness relative to PA138 and PA109.

8.3 Discussion.

In this chapter experiments demonstrating that a subset of *P. aureofaciens* PA147-2 antifungal defective mutants has an *in vitro* competitive advantage over the wildtype are presented. The two members of this group appear to have mutations in genes that encode global regulators of antifungal activity, whereas the mutations in the non-competitive class are likely to have more specific effects. Why do PA138 and PA109 appear to have increased fitness relative to the parental strain? Previous research has demonstrated that the bleomycin resistance gene of Tn*5* can confer a growth advantage on *E. coli* (Blot et al., 1994). However, this is an unlikely explanation for the results presented here because only two of the five Tn*5*-containing strains showed increased fitness. If the mere presence of Tn*5* was sufficient to confer a growth advantage, all five Tn*5*-generated mutants would be expected to outcompete PA147-*2lacZY* with equal success. Since this was not observed it

is reasonable to conclude that the competitive phenotypes are a consequence of the specific mutations in PA109 and PA138. Transposition of Tn*5* to produce fitter mutants is also unlikely, based upon Southern analysis of strains from starved cultures. Based upon two observations it appears that it is also unlikely that incorporation of the *lacZY* on Tn*7* was responsible for the reduced fitness of the wildtype seen in competition with group 2 mutants. Firstly, if introduction of Tn*7lacZY* resulted in a wildtype of inherently lower fitness one would expect the group 1 mutants to be as competitive as the group 2 mutants. Secondly, the competition and bioassay experiments did not reveal any differences between PA147-2 and PA147-2*lacZY* under relevant conditions, supporting the neutrality of the introduced marker. This is in agreement with experiments on *P. fluorescens* SBW25 suggesting that, under some conditions, the addition of *lacZY* genes had no effect on the fitness of this strain which had been previously modified by the incorporation of *aph-1* (Km^R) and *xylE* (catechol 2,3-dioxygenase) (De Leij et al., 1998). Finally, the analysis of growth rates indicates that PA138 and PA109 have a similar exponential growth rate to PA147-2*lacZY*. Thus, the group 2 mutants do not have an obvious advantage in the first hours of the competition experiments. Further to this, the fact that PA147-2*lacZY* does not start to "lose" the competition until between the second and third days rules out differences in generation time as a possible explanation.

8.3.1 PA109 and PA138 behave like GASP mutants.

The competitive phenotype displayed by PA109 and PA138 strongly resembles the GASP phenotype of *E. coli*. Both mutants show increased fitness relative to the wildtype, and even when inoculated as a minority population, PA138 was sufficiently fit to take over the culture. Thus, it appears that the fitness increase of PA138 and PA109 might not be due to being defective for antifungal activity *per se*, but rather the pleiotrophic effects of the global regulatory mutations confers a GASP-like phenotype. The correlation of global regulation with increased fitness strengthens the relevance of this work to GASP studies, since mutations in the global regulator genes *rpoS* and *lrp* have both been implicated in the GASP phenotype in *E. coli* (Zambrano et al., 1993; Zinser & Kolter, 2000). However, to unequivocally ascribe the GASP-like effect to the Tn*5* mutations in PA109 and PA138, complementation experiments need to be carried out. It is possible that PA138 and PA109 have coincidentally both acquired a secondary mutation that confers increased fitness.

PA109 and PA138 that have both been restored for fungal inhibition by allele exchange (Carruthers et al., 1994; chapter 5), and use of these strains in competition would reveal whether the Tn*5* insertion is the genuine origin of the competitive phenotype. Our observation that aged cultures of PA147-2*lacZY* are more competitive with PA138 and PA109 than the parental (not starved) strain suggest that, as is the case in *E. coli*, fitter (GASP) strains arise in aged cultures of *P. aureofaciens*. The increased fitness is also accompanied by loss or reduction of antifungal activity by a large number of strains isolated after 20 days of starvation. This observation provides indirect support for the notion that the group 2 antifungal mutants might be akin to GASP strains. In addition, these results suggest that under stressful conditions (e.g. starvation), reducing or eliminating antifungal activity can result in enhanced survival of *P. aureofaciens*, possibly by reducing the metabolic burden of synthesizing an antifungal metabolite. The fact that the replicates of starvation cultures of PA147-2 have variation between them supports the suggestion that the loss or reduction of antifungal activity results from mutations arising in culture, and the difference between them could be explained by beneficial mutations which arise early or late in the experiment. Zinser and Kolter (2000) proposed that mutations in regulator genes are highly beneficial because they result in the simultaneous alteration of multiple cellular activities. PA109 and PA138 are regulatory mutants and appear to support their proposal. The addition of mutations in two-component and lysR-type regulators to the spectrum of potential GASP alleles strengthens the hypothesis that fitter strains can emerge as a result of the coordinated repression of a large number of genes (Zinser & Kolter, 2000), and the observation of a GASP-like phenotype in a non *E. coli* system increases the relevance of GASP research to the study of microbial evolution. Future analysis aimed at determining whether the antifungal mutants that arose in 20-day cultures have mutations in genes for global regulation of fungal inhibition, and whether they show increased fitness in competition with PA109 and PA138 would be of great interest. Such observations would support the suggestion that some regulatory mutations are favoured by selection because of the benefits to fitness of reducing expression of an entire regulon (Zinser & Kolter, 2000).

8.3.2 Culture takeover by PA109 and PA138 is not complete.

PA138 and PA109 do not drive PA147-2*lacZY* to extinction. Superficially, this observation is in contrast to many GASP experiments in which the less fit strain becomes

undetectable by the end of the experiment (Finkel & Kolter, 1999; Zinser & Kolter, 1999). However, over longer term experiments the "rise and fall" of different strains can be observed as conditions change (Finkel & Kolter, 1999). Even those strains that fell below the threshold of detection could revive. It is thus possible that if conditions were to change, PA138 and PA109 may lose their competitive advantage, allowing PA147-2*lacZY* to recover. However, given that antifungal defective mutants arise in monoculture, we speculate that even if PA147-2*lacZY* recovered as conditions changed, it is possible that at least some of those "wildtype" strains that recover will have acquired mutations that render them defective for fungal inhibition.

8.3.3 Implications for biocontrol?

Although these experiments were entirely *in vitro*, it is worth considering the potential importance of the observations in the application of biocontrol agents in the field, given that stresses such as starvation are likely to be commonplace in the soil environment. In this regard, our results would suggest that PA147-2 might not be an ideal biocontrol agent. However, detailed inspection of the mode of application (e.g. seed coating, pellet formulations) of biocontrol bacteria, and the affinity of PA147-2 for plant root attachment (R.D. Monds and H.K. Mahanty, unpublished data) indicate that PA147-2 and similar bacteria can still be useful in combating fungal disease in plants. The methods of application aid in targeting the bacteria to the root, where the environment encountered is considerably different to the more stressful conditions found in soil. From the work presented it is clear that under starvation conditions, antifungal minus and reduced mutants can arise and persist and two antifungal mutants (PA109 and PA138) have the ability to outcompete PA147-2*lacZY*. The competitive advantage of some antifungal mutants suggests that if this kind of mutant should arise in the soil, it could be accompanied by a concomitant loss of biocontrol efficacy as the mutant population increases at the expense of the wildtype. However, a recent study by Mirleau et al. (2000) has shown that a mutant of *P. fluorescens* C7R12 that is defective in pyoverdine synthesis (designated PL1) and is less fit than the parental strain in competition experiments under gnotobiotic conditions, shows similar competitive fitness to the wildtype in non-gnotobiotic soil experiments. Since pyoverdine has been implicated in biocontrol this study provides some insight into the contribution of biocontrol factors on competitive fitness. Pyoverdine is a siderophore,

which are compounds involved in iron scavenging (Neilands, 1981), and thus thought to contribute to biocontrol efficacy by reducing iron availability for the fungal pathogen rather than antibiosis *per se* (Leong, 1986). Mirleau et al (Mirleau et al., 2000) suggest that the ability to compete well in non-gnotobiotic conditions could be a consequence of the ability of *P. fluorescens* C7R12 and PL1 to uptake siderophores produced by other organisms. This would result in PL1 being able to scavenge enough iron as long as other siderophore producing organisms were present.

Studies on phenazine production indicate that producing the antibiotic is a factor in the ecological success of *P. fluorescens* 2-79 and *P. aureofaciens* 30-84 (Mazzola et al., 1992). This was thought to be because the ability to kill competitors provides a competitive advantage when nutrients are limited. Phenazines would be very effective compounds in that regard, given that they have antibacterial properties in addition to their antifungal activity. This is in contrast to PA147-2, which has never been observed to inhibit the growth of bacteria *in vitro*. Thus, production of antibiotics by PA147-2 would have limited success in excluding all competitors, since bacteria would most likely be unaffected. Since our mutants PA109 and PA138 are deficient in production of an exclusively antifungal metabolite, and the experimental system lacks other competing species, it is unclear whether the complex environment of soil would lead to observations similar to those of Mazzola et al (1992) or Mirleau et al (2000), or whether our starvation experiments accurately simulate events that could take place in soil. Furthermore, although starvation conditions may be encountered in the soil, the environment on and adjacent to plant roots (the rhizosphere) may be supplied with nutrients (sugars and amino acids) from root exudates (Jaeger et al., 1999). Thus, it is possible that starvation may be a rare or sporadic occurrence in the rhizosphere, which is also the site of action for biocontrol agents used in the suppression of root diseases. Therefore, an important distinction may exist between PA147-2 that is active in biocontrol and has colonized the rhizosphere, and those cells that are left to persist in the soil once crops have been removed. If this is the case, cells remaining between crop plantings could be the most likely to endure starvation and accumulate GASP mutations, making their long-term reliability questionable.

As discussed in chapter 7, during the field trial PA147-2 in bulk soil reduced to undetectable levels, whereas in the rhizosphere PA147-2 persisted for the duration of the study, albeit at low levels. The observed difference may support the suggestion (above) that it is the cells in bulk soil that may be the least likely to survive. Whether the lack of survival in soil indicates that PA147-2 will not persist by acquiring GASP mutations is uncertain as the conditions used to induce *Phytophthora* rot are reasonably harsh, involving flooding followed by water stress. A trial that requires less severe treatments and provides a better chance for rhizosphere colonisation would provide better data in this regard. An example would be the control of take-all of wheat. In this system, bacterial seed coating could be used, which is likely to be better than dipping roots in a bacterial suspension, with regard to colonisation efficacy. These intriguing and testable questions could be addressed by comparisons between soil microcosm and rhizosphere experiments under controlled conditions, followed by further fieldwork. Such work would go a long way to addressing the question of whether soil bacteria accumulate GASP mutations as a survival strategy, and whether such processes have implications for the selection of biocontrol bacteria.

In summary, I have demonstrated that global regulatory mutations can improve the competitive fitness of *P. aureofaciens* PA147-2 in a manner similar to the GASP phenotype in *E. coli*. These observations are of interest for the study of both microbial evolution and biological control. Future experiments will focus on the questions arising from our *in vitro* starvation experiments from which spontaneous antifungal mutants were recovered. I am interested in examining the fitness of individual clones from the starved populations, and testing the hypothesis that they have mutations in global regulators of antifungal activity. These experiments could then be extended into systems that model field conditions.

Chapter 9

Summary of results, comments, and future directions

The work presented in this thesis was undertaken with a view to furthering the understanding of how *P. aureofaciens* PA147-2 inhibits fungal growth, and increasing the knowledge of the usefulness of PA147-2 as a biocontrol agent. Research results are presented in six chapters (Chapters 3-8), and although at first glance these appear to be unrelated, this is not so when the whole body of work is considered in detail. Below I assess the relative success of the work presented in these chapters, show how the chapters are related, and summarise the novel findings of the research.

An analysis of the *recA* gene in PA147-2 was undertaken to develop a tool for use in the study of PA147-2. Construction of a simple system for the introduction of *recA* mutations would be of great value in the study of PA147-2 mutants. In Chapter 3, the successful development of a *recA* deletion system was described, and this system was employed in the complementation of the mutation in PA109 (Chapter 5). In addition, a seemingly novel function of *recA* was uncovered in this study. In PA147-2, it appears that *recA* is involved in the observed instability of the pLAFR3 cosmid vector, and cosmids constructed using pLAFR3. The introduction of the *recA*1 mutation increased stability of a cosmid, while the *recA*4 mutation had no significant effect on cosmid stability. Both mutations rendered PA147-2 sensitive to UV and MMS induced DNA damage. Thus, the investigation of *recA* was successful, and relates to other aspects of this thesis in that it led to the creation of a tool that was subsequently used in this study of PA147-2. Furthermore, the study of *recA* resulted in a publication in the Canadian Journal of Microbiology (Silby & Mahanty, 2000) (appendix 5).

Three chapters present results pertaining to the study of antifungal mutants in PA147-2. In Chapter 4, a range of mutants were subjected to a preliminary analysis, which identified a number of potentially important genes for antifungal activity. Apart from the identification of two putative global regulators, the genes found were not similar to those normally associated with antifungal activity. Potential roles for these genes were discussed. The

results presented in Chapter 4 were important for the rest of this thesis in that these results provided the initial information required for the studies detailed in Chapters 5 and 6. The genes identified were also important in discussion and interpretation of the results of the competition experiments described in Chapter 8. The major limitation of the work outlined in chapter 4 was the failure to identify a gene or gene cluster required for the biosynthesis of antifungal metabolites, as found in other *Pseudomonas* spp. biocontrol strains. Since characterisation by chemical means has been unsuccessful (Godfrey, 1997), it was hoped that a molecular approach would be useful in the identification of the compound(s) responsible for fungal inhibition. One possible remedy for this situation is the creation of further mutants. The original antifungal mutants were created using a wildtype Tn*5*, but the isolation of large numbers of mutants was relatively difficult (F. Carruthers, Pers. Comm.). Recently, miniTn*5* derivatives (De Lorenzo et al., 1990) have been successfully used in PA147-2 for the isolation of biofilm-defective mutants (Monds, 2000). Thus, the use of miniTn*5* elements may facilitate the isolation of additional antifungal mutants, and DNA sequencing of insertion points might permit the identification of biosynthetic genes.

Chapter 5 describes experiments that were used to study two putative regulators of antifungal activity. One of these, designated FinR, had similarity to LysR-type transcriptional regulators, while the other (FinT) was similar to hybrid two-component sensor/regulators. When present *in cis*, FinR was shown to affect expression of an adjacent gene (*finA*), which was also involved in antifungal activity. In addition, the results suggested that FinR had other regulatory targets that were important for antifungal activity. This is a novel finding, given that most global regulators of antifungal activity appear to be of the two-component type. FinT appears to be a two-component regulator of antifungal activity, but does not seem to be a homologue of the GacA/GacS two-component regulator often described in biocontrol pseudomonads. It seems that FinT and FinR share regulatory targets, as revealed by examination of their protein profiles relative to the wildtype. Common proteins are missing in the *finR* and *finT* mutants. Interestingly, the influence of FinT appears to be dependent upon extracellular inorganic phosphate concentration. Experiments in Chapter 6 show that the *finT* mutant can be antifungal positive in the presence of high Pi, lending support to a model for phosphate-regulated fungal inhibition in PA147-2. It would be interesting to examine the protein profile of PA109 grown in the

presence of 25mM Pi. Given the antifungal phenotype under Pi excess, the protein profile would be predicted to resemble PA147-2, rather than PA138 or PA109 grown in limited Pi conditions. FinR appears to be unaffected by Pi, an observation that provides support to the suggestion that FinR operates near the top of the antifungal regulatory hierarchy. Additional experiments presented in Chapter 6 suggested that the phosphate specific transport (Pst) system has an influence on the expression of antifungal activity in low phosphate conditions. Thus it appears that there may be an important link between the regulator FinT, and the Pst system with respect to inhibition of fungi in low phosphate environments. Further experiments to examine FinT-regulated genes under low and high phosphate conditions would be revealing. Such studies, using 2-D protein separations, or even DNA arrays could allow the isolation of biosynthetic genes controlled by FinT, or provide information regarding the possible link between FinT and the Pst system. Additionally, if FinR was included, a picture of regulatory hierarchy could emerge. Finally, studies of *finT* fused to reporter genes would be of interest because the expression of FinT could be monitored in different mutant backgrounds (e.g. *finR*, *pstA*), and with different phosphate concentrations in the growth media. This type of experiment would indicate whether Pst affects *finT* or vice versa, whether *finT* is controlled in some way by FinR, and whether phosphate influences expression of FinT.

The remaining experimental chapters are important in the assessment of the applicability of PA147-2 as a biocontrol agent. In Chapter 7 the results of a field trial are presented. This was a preliminary trial, and therefore was limited in its size and scope. Despite this, it showed that PA147-2 provided significant protection from *Phytophthora* rot to asparagus plants. While this protection was relatively limited compared to chemical controls, the fact that it was significant relative to untreated controls suggests PA147-2 is a strain worthy of further study. It is possible that the results would be considerably better if the trial design was improved. For example, the method of applying the bacterial treatment was crude, and could have easily contributed to the declining population of PA147-2 that was evident in the trial. More sophisticated application techniques might give the bacteria a better chance at survival.

The observation that the PA147-2 population did not establish at a stable level led to the experiments shown in Chapter 8. Previous studies on the biocontrol strains *P. fluorescens* 2-79 and *P. aureofaciens* 30-84 had implicated phenazine antibiotic production as an important factor in the competitive fitness of the organism in soil (Mazzola et al., 1992). The fact that PA147-2 was unable to establish a population in the field raised the question of whether antibiotic production was a burden for this strain. This was addressed *in vitro* in Chapter 8. The hypothesis was that if producing antifungal compounds was a burden, mutants that do not produce those compounds would outcompete the wildtype. The results indicated that global regulatory mutants have increased relative fitness, and this trait resembled the GASP phenomenon known in *E. coli*. This study provided evidence that PA147-2 could mutate to an antifungal-deficient form, and that these mutants could survive and proliferate in long term cultures. These experiments provide a strong rationale for further examination of PA147-2 fitness, under more complex conditions such as in soil microcosms. They also lead to a cautionary note on the use of PA147-2 in that perhaps this strain will not establish high populations because of relatively low fitness, or alternatively, in the absence of fungal pathogens PA147-2 will mutate to a fitter, antifungal-defective variant, resulting in reduced biocontrol efficacy. It would be intriguing to examine the fitness of PA147-2 relative to the seemingly fitter mutants PA109 and PA138 under more natural conditions, and competition experiments against other soil pseudomonads would provide valuable data on the relative fitness against the type of organism PA147-2 would encounter in a biocontrol situation.

As has been outlined, the several lines of research presented in this thesis are intimately linked. The early chapters (3 and 4) provided invaluable tools and resources for the molecular studies (Chapters 5 and 6). The analysis of PA109 and PA138 as regulatory mutants (Chapter 5) led to the tests of FinR and FinT as candidates for a hypothetical low-phosphate regulator in Chapter 6. Putative identification of the genes interrupted by transposons, described in Chapters 4, 5 and 6, allowed suggestions to be made in Chapter 8 regarding competitive fitness, and the possibility that regulatory mutants were fitter than non-regulatory mutants and the wildtype. The experiments carried out in Chapter 8 were initiated as a result of the field trial (Chapter 7), and the observed population decline of PA147-2 which suggested low competitive fitness.

Future directions.

The work described has opened a number of avenues for future research, all of which would add greatly to the growing understanding of PA147-2 as a biocontrol strain. The studies on global regulation have shown that there are a number of differences that PA138 and PA109 share compared to PA147-2. An intensive study on the proteins that PA138 and PA109 cannot synthesis would reveal at least some of their regulatory targets, which could be involved in antifungal activity. Having identified the proteins, it would be possible to identify and mutate the gene, allowing its role in fungal inhibition to be tested. As a complement to that study, it would be interesting to revisit the chemical nature of the antifungal compound(s). Although it has proven difficult to study, the identification of the compound(s) is perhaps the single most pressing need for the future study of PA147-2. Identification of the compound(s) will allow hypotheses to be made and tested regarding the biosynthetic pathway(s). Furthermore, simple purification procedures might open possibilities to study production *in situ*, and to address questions regarding its stability in soil. All of this is important for the application of PA147-2 as a biocontrol agent. As mentioned above, soil microcosm studies will more accurately assess the fitness of PA147-2 compared to a range of bacteria. Another important question to arise from this study is the nature of the link between antifungal compound production, phosphate concentration, and biofilm formation. Clearly there is a complex regulatory system involved, possibly using the Pst system as an environmental sensor. Further elucidation of the role of phosphate in controlling both fungal inhibition and biofilm formation will be of value from fundamental and applied points of view.

Although there are many directions that have been opened by this work, it seems that the future research must also involve the generation of new mutants. The present collection is small, and does not seem to give clues regarding the biosynthetic genes. Thus, a new mutant hunt would be a useful undertaking, particularly if the chemical characterisation of the antifungal compound is also undertaken. Together these approaches might finally reveal the nature of the compound produced by PA147-2.

Summary statement.

At the start of this chapter the broad goals of "furthering the understanding of how *P. aureofaciens* PA147-2 inhibits fungal growth, and increasing the knowledge of the usefulness of PA147-2 as a biocontrol agent" were mentioned. This work has shown that two global regulators are involved in antifungal activity, one of which appears to be required only under low phosphate conditions. The phosphate specific transport system has been implicated in low-phosphate fungal inhibition, probably via a mechanism unrelated to its role in phosphate assimilation and metabolism. A tantalising link between antifungal activity and biofilm formation also emerged from this study. A number of mutants were subjected to preliminary characterisation, and these results suggest a range of physically unlinked genes are required for fungal inhibition. A field trial demonstrated that PA147-2 can suppress *Phytophthora* rot of asparagus, and that PA147-2 could not establish a stable population. *In vitro* experiments provided some evidence that antibiotic production by PA147-2 is metabolically costly, and that fitter mutants can arise during starvation culture. Thus, while there is clearly a considerable amount of work to be done on PA147-2, the investigations presented in this thesis have successfully increased the understanding of the molecular mechanisms that contribute to fungal inhibition by PA147-2, and demonstrated the potential of PA147-2 as a biocontrol candidate.

References

Altschul, S. F., Gish, W., Miller, W., Myers, E. W. & Lipman, D. J. (1990). Basic local alignment search tool. *Journal of Molecular Biology* **215**, 403-10.

Altschul, S. F., Madden, T. L. & Schaffer, A. A. (1997). Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Research* **25**, 3389-3402.

Anderson, A. J. (1983). Isolation from root and shoot surfaces of agglutinins that show specificity for saprophytic Pseudomonads. *Canadian Journal of Botany* **61**, 3438-3443.

Anderson, A. J., Tari, P. H. & Tepper, C. S. (1988). Molecular studies on the role of a root surface agglutinin in adherence and colonization by *Pseudomonas putida*. *Applied and Environmental Microbiology* **54**, 375-380.

Argast, M., Ludtke, D., Silhavy, T. J. & Boos, W. (1978). A second transport system for sn-glycerol-3-phosphate in *Escherichia coli*. *Journal of Bacteriology* **136**, 1070-1083.

Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A. & Struhl, K. (1989). *Short protocols in molecular biology*. New York: John Wiley and Sons Inc.

Baker, K. F. (1987). Evolving concepts of biological control of plant pathogens. *Annual review of Phytopathology* **25**, 67-85.

Baker, R. (1968). Mechanisms of biological control of soil-borne pathogens. *Annual review of Phytopathology* **6**, 263-294.

Ball, T. K., Wasmuth, C. R., Braunagel, S. C. & Benedik, M. J. (1990). Expression of *Serratia marcescens* extracellular proteins requires *recA*. *Journal of Bacteriology* **172**, 342-349.

Bangera, M. G. & Thomashow, L. S. (1996). Characterisation of a genomic locus required for synthesis of the antibiotic 2,4-diacetylphloroglucinol by the biological control agent *Pseudomonas fluorescens* Q2-87. *Molecular Plant Microbe Interactions* **9**, 83-90.

Bangera, M. G. & Thomashow, L. S. (1999). Identification and characterization of a gene cluster for synthesis of the polyketide antibiotic 2,4-diacetylphloroglucinol from *Pseudomonas fluorescens* Q2-87. *Journal of Bacteriology* **181**, 3155-3163.

Barry, G. F. (1988). A broad-host -range shuttle system for gene insertion into the chromosomes of gram-negative bacteria. *Gene* **71**, 75-84.

Belanger, M., Burrows, L. L. & Lam, J. S. (1999). Functional analysis of genes responsible for the synthesis of the B-band O antigen of *Pseudomonas aeruginosa* serotype O6 lipopolysaccharide. *Microbiology* **145**, 3505-3521.

Bender, C. L., Rangaswamy, V. & Loper, J. (1999). Polyketide production by plant-associated pseudomonads. *Annual review of Phytopathology* **37**, 175-196.

Berger, K. C. (1965). *Introductory soils*. New York: The Macmillan company.

Better, M. & Helinski, D. R. (1983). Isolation and characterisation of the *recA* gene of *Rhizobium meliloti*. *Journal of Bacteriology* **155**, 311-316.

Bi, E. & Lutkenhaus, J. (1993). Cell division inhibitors SulA and MinCD prevent formation of the FtsZ ring. *Journal of Bacteriology* **175**, 1118-1125.

Blehert, D. S., Fox, B. G. & Chambliss, G. H. (1999). Cloning and sequence analysis of two *Pseudomonas* flavoprotein xenobiotic reductases. *Journal of Bacteriology* **181**, 6254-6263.

Blehert, D. S., Knoke, K. L., Fox, B. G. & Chambliss, G. H. (1997). Regioselectivity of nitroglycerin denitration by flavoprotein nitroester reductases purified from two *Pseudomonas* species. *Journal of Bacteriology* **179**, 6912-6920.

Blot, M., Hauer, B. & Monnet, G. (1994). The Tn*5* bleomycin resistance gene confers improved survival and growth advantage on *Escherichia coli*. *Molecular and General Genetics* **242**, 595-601.

Blumer, C. & Haas, D. (2000a). Mechanism, regulation, and ecological role of bacterial cyanide biosynthesis. *Archives of Microbiology* **173**, 170-177.

Blumer, C. & Haas, D. (2000b). Multicopy supression of a *gacA* mutation by the *infC* operon in *Pseudomonas fluorescens* CHAO: competition with the global translational regulator RsmA. *FEMS Microbiology Letters* **187**, 53-58.

Blumer, C., Heeb, S., Pessi, G. & Haas, D. (1999). Global GacA-steered control of cyanide and exoprotease production in *Pseudomonas fluorescens* involves specific ribosome binding sites. *Proceedings of the National Academy of Sciences of the United States of America* **96**, 14073-14078.

Bolivar, F., Rodriguez, R. L., Greene, P. J., Betlach, M. C., Heyneker, H. L., Boyer, H. W., J.H, C. & Falkow, S. (1977). Construction and characterisation of new cloning vehicles II. A multipurpose cloning system. *Gene* **2**, 95-113.

Botero, L. M., Al-Niemi, T. S. & McDermott, T. R. (2000). Characterization of two inducible phosphate transport systems in *Rhizobium tropici*. *Applied and Environmental Microbiology* **66**, 15-22.

Bowers, J. H. & Parke, J. L. (1993). Colonization of pea (*Pisum sativum* L.) taproots by *Pseudomonas fluorescens*: effect of soil temperature and bacterial motility. *Soil Biology and Biochemistry* **25**, 1693-1701.

Brockman, F. J., Forse, L. B., Bezdicek, D. F. & Fredrickson, J. K. (1991). Impairment of transposon-induced mutants of *Rhizobium leguminosarum*. *Soil Biology and Biochemistry* **23**, 861-868.

Brzoska, P., Rimmele, M., Brzostek, K. & Boos, W. (1994). The pho regulon-dependent Ugp uptake system for glycerol-3-phosphate in *Escherichia coli* is *trans* inhibited by Pi. *Journal of Bacteriology* **176**, 15-20.

Buckman, H. O. & Brady, N. C. (1969). *The nature and properties of soils*, seventh edn. New York: The Macmillan company.

Bull, C. T., Weller, D. M. & Thomashow, L. S. (1991). Relationship between root colonization and suppression of *Gaeumannomyces graminis* var. *tritici* by *Pseudomonas fluorescens* strain 2-79. *Phytopathology* **81**, 954-959.

Burkhead, K. D., Schisler, D. A. & Slininger, P. J. (1994). Pyrrolnitrin production by biological control agent *Pseudomonas cepacia* B37w in culture and in colonized wounds of potatoes. *Applied and Environmental Microbiology* **60**, 2031-2039.

Carlile, M. J. (1983). Motility, taxis, and tropism in *Phytophthora*. In *Phytophthora: Its biology, taxonomy, ecology, and pathology*, pp. 95-107. Edited by D. C. Erwin, S. Bartnicki-Garcia & P. H. Tsao. Minnesota: The American Phytopathological Society.

Carruthers, F. L. (1994). A study of antifungal activity by a potential biological control strain, *Pseudomonas aureofaciens* strain PA147-2. In *Department of Plant and Microbial Sciences*, pp. 141. Christchurch, New Zealand: University of Canterbury.

Carruthers, F. L., Conner, A. J. & Mahanty, H. K. (1994). Identification of a Genetic Locus in *Pseudomonas aureofaciens* involved in fungal inhibition. *Applied and Environmental Microbiology* **60**, 71-77.

Carruthers, F. L., Shum-Thomas, T., Conner, A. J. & Mahanty, H. K. (1995). The significance of antibiotic production by *Pseudomonas aureofaciens* PA147-2 for biological control of *Phytophthora megasperma* root rot of asparagus. *Plant and Soil* **170**, 339-344.

Chan, F. Y. & Torriani, A. (1996). PstB protein of the phosphate-specific transport system of *Escherichia coli* is an ATPase. *Journal of Bacteriology* **178**, 3974-3977.

Chancey, S. T., Wood, D. W. & Pierson, L. S. (1999). Two-component transcriptional regulation of N-acyl-homoserine lactone production in *Pseudomonas aureofaciens*. *Applied and Environmental Microbiology* **65**, 2294-2299.

Chang, A. C. Y. & Cohen, S. N. (1978). Construction and characterization of amplifiable multicopy DNA cloning vehicles derived from p15a cryptic miniplasmid. *Journal of Bacteriology* **134**, 1141-1156.

Chernin, L., Brandis, A., Ismailov, Z. & Chet, I. (1996). Pyrrolnitrin production by an *Enterobacter agglomerans* strain with a broad spectrum of antagonistic activity towards fungal and bacterial phytopathogens. *Current Microbiology* **32**, 208-212.

Clark, A. J. & Margulies, A. D. (1965). Isolation and characterisation of recombination-deficient mutants of *E. coli* K12. *Proceedings of the National Academy of Sciences USA* **53**, 451-459.

Clough, S. J., Flavier, A. B., Schell, M. A. & Denny, T. P. (1997). Differential expression of virulence genes and motility in *Ralstonia* (*Pseudomonas*) *solanacearum* during exponential growth. *Applied and Environmental Microbiology* **63**, 844-850.

Comstock, L. E., Johnson, J. A., Michalski, J. M., Morris, J. G. & Kaper, J. B. (1996). Cloning and sequence of a region encoding a surface polysaccharide of *Vibrio cholerae* O139 and characterization of the insertion site in the chromosome of *Vibrio cholerae* O1. *Molecular Microbiology* **19**, 815-826.

Cook, R. J. & Rovira, A. D. (1976). Role of bacteria in biological control of *Gaeumannomyces graminis* by suppressive soils. *Soil Biology and Biochemistry* **8**, 269-273.

Corbell, N. & Loper, J. E. (1995). A global regulator of secondary metabolite production in *Pseudomonas fluorescens* Pf-5. *Journal of Bacteriology* **177**, 6230-6236.

Coschigano, P. W. & Young, L. Y. (1997). Identification and sequence analysis of two regulatory genes involved in anaerobic toluene metabolism by strain T1. *Applied and Environmental Microbiology* **63**, 652-660.

Cox, G. B., Webb, D., Godovac-Zimmermann, J. & Rosenberg, H. (1988). Arg-220 of the PstA protein is required for phosphate transport through the phosphate-specific transport system in *Escherichia coli* but not for alkaline phosphatase repression. *Journal of Bacteriology* **170**, 2283-2286.

Cox, G. B., Webb, D. & Rosenberg, H. (1989). Specific amino acid residues in both the PstB and PstC proteins are required for phosphate transport by the *Escherichia coli* Pst system. *Journal of Bacteriology* **171**, 1531-1534.

Daigle, F., Fairbrother, J. M. & Harel, J. (1995). Identification of a mutation in the *pst-phoU* operon that reduces pathogenicity of an *Escherichia coli* strain causing septicemia in pigs. *Infection and Immunity* **63**, 4924-4927.

Davey, M. E. & O'Toole, G. A. (2000). Microbial biofilms: from ecology to molecular genetics. *Microbiology and Molecular Biology Reviews* **64**, 847-867.

Davidse, L. C. (1984). Antifungal activity of acylalanine fungicides and related chloroacetanilide herbicides. In *Mode of Action of Antifungal Agents*, pp. 239-255. Edited by A. P. J. Trinci & J. F. Riley. Cambridge: Cambridge University Press.

De Leij, F. A. A. M., Thomas, C. E., Bailey, M. J., Whipps, J. M. & Lynch, J. M. (1998). Effect of insertion site and metabolic load on the environmental fitness of a genetically modified *Pseudomonas fluorescens* isolate. *Applied and Environmental Microbiology* **64**, 2634-2638.

De Lorenzo, V., Herrero, M., Jakubzik, U. & Timmis, K. N. (1990). Mini-Tn*5* transposon derivatives for insertion mutagenesis, promoter probing, and chromosomal insertion of cloned DNA in gram negative eubacteria. *Journal of Bacteriology* **172**, 6568-6572.

De Mot, R., Laeremans, T., Schoofs, G. & Vanderleyden, J. (1993). Characterization of the *recA* gene from *Pseudomonas fluorescens* OE 28.3 and construction of a *recA* mutant. *Journal of General Microbiology* **139**, 49-57.

de Weger, L. A., van der Vlugt, C. I. M., Wijfjes, A. H. M., Bakker, P. A. H. M., Schippers, B. & Lugtenberg, B. J. (1987). Flagella of a plant-growth-stimulating *Pseudomonas fluorescens* strain are required for colonization of potato roots. *Journal of Bacteriology* **169**, 2769-2773.

Debbia, E. A. (1992). Filamentation promotes F'*lac* loss in *Escherichia coli* K12. *Journal of General Microbiology* **138**, 2083-2091.

Delaney, S. M., Mavrodi, D. V., Bonsall, R. F. & Thomashow, L. S. (2001). *phzO*, a gene for biosynthesis of 2-hydroxylated phenazine compounds in *Pseudomonas aureofaciens* 30-84. *Journal of Bacteriology* **183**, 318-327.

Delany, I., Sheehan, M. M., Fenton, A., Bardin, S., Aarons, S. & O'Gara, F. (2000). Regulation of production of the antifungal metabolite 2,4-diacetylphloroglucinol in *Pseudomonas fluorescens* F113: genetic analysis of *phlF* as a transcriptional repressor. *Microbiology* **146**, 537-546.

Duffy, B. K. & Defago, G. (1999). Environmental factors modulating antibiotic and siderophore biosynthesis by *Pseudomonas fluorescens* biocontrol strains. *Applied and Environmental Microbiology* **65**, 2429-2438.

Ennis, D. G., Ossanna, N. & Mount, D. W. (1989). Genetic separation of *Escherichia coli recA* functions for SOS mutagenesis and repressor cleavage. *Journal of Bacteriology* **171**, 2533-2541.

Falloon, P. G. (1991). Effect of root temperature, plant age, frequency and duration of flooding and inoculum placement and concentration on susceptibility of asparagus to *Phytophthora* rot. *New Zealand Journal of Crop and Horticultural Science* **19**, 305-312.

Falloon, P. G., Benson, B. L. & Grogan, R. G. (1986). Effect of *Phytophthora megasperma* var *sojae* on yield *Asparagus officinalis*. *Plant Disease* **70**, 103-105.

Falloon, P. G., Greathead, A. S., Mullen, R. J., Benson, B. L. & Grogan, R. G. (1991). Individual and combined effects of flooding, Phytophthora rot, and Metalaxyl on asparagus establishment. *Plant Disease* **75**, 514-518.

Falloon, P. G., Mullen, R. J., Benson, B. L. & Grogan, R. G. (1985). Control of *Phytophthora* rot with metalaxyl in established asparagus. *Plant Disease* **69**, 921-923.

Falloon, P. G. & Tate, K. G. (1986). Major diseases of asparagus in New Zealand. *Proceedings of the agronomy society of New Zealand.* **16**, 17-28.

Figiel, R. (1994). Vintners take the pledge - Grape growers and wine makers switch from using chemical pesticides to organic farming methods. *Sierra* **79**, 51-5.

Finkel, S. E. & Kolter, R. (1999). Evolution of microbial diversity during prolonged starvation. *Proceedings of the National Academy of Sciences USA* **96**, 4023-4027.

Forst, S., Delgado, J. & Inouye, M. (1989). Phosphorylation of OmpR by the osmosensor EnvZ modulates expression of the *ompF* and *ompC* genes in *Escherichia coli*. *Proceedings of the National Academy of Sciences of the United States of America* **86**, 6052-6056.

Fournier, B. & Hooper, D. C. (2000). A new two-component regulatory system involved in adhesion autolysis, and extracellular proteolytic activity of *Staphylococcus aureus*. *Journal of Bacteriology* **182**, 3955-3964.

Fravel, D. R. (1988). Role of antibiosis in the biocontrol of plant diseases. *Annual review of Phytopathology* **26**, 75-91.

French, C. E., Nicklin, S. & Bruce, N. C. (1996). Sequence and properties of pentaerythritol tetranitrate reductase from *Enterobacter cloacae* PB2. *Journal of Bacteriology* **178**, 6623-6627.

French, C. E., Nicklin, S. & Bruce, N. C. (1998). Aerobic degradation of 2,4,6-trinitrotoluene by *Enterobacter cloacae* PB2 and by pentaerythritol tetranitrate reductase. *Applied and Environmental Microbiology* **64**, 2864-2868.

Gaffney, T. D., Lam, S. T., Ligon, J., Gates, K., Frazelle, A., Di Maio, J., Hill, S., Goodwin, S., Torkewitz, N., Allshouse, A. M., Kempf, H. J. & Becker, J. O. (1994). Global regulation of expression of antifungal factors by a *Pseudomonas fluorescens* biological control strain. *Molecular Plant Microbe Interactions* **7**, 455-463.

Gilson, L., Mahanty, H. K. & Kolter, R. (1987). Four plasmid genes are required for colicin V synthesis, export and immunity. *Journal of Bacteriology* **169**, 2466-2470.

Glandorf, D. C. M., Van Der Sluis, I., Anderson, A. J., Bakker, P. A. H. M. & Schippers, B. (1994). Agglutination, adherence, and root colonization by fluorescent pseudomonads. *Applied and Environmental Microbiology* **60**, 1726-1733.

Godfrey, S. A. C. (1997). Further investigation of the antifungal activity shown toward *Phytophthora megasperma* var. *sojae* by the potential biological control strain *Pseudomonas aureofaciens* PA147-2. In *Department of Plant and Microbial Sciences*, pp. 163. Christchurch, New Zealand: University of Canterbury.

Godfrey, S. A. C., Silby, M. W., Falloon, P. G. & Mahanty, H. K. (2000). Biological control of *Phytophthora megasperma* var. *sojae*, causal agent of Phytophthora rot of asparagus, by *Pseudomonas aureofaciens* PA147-2: a preliminary field trial. *New Zealand Journal of Crop and Horticultural Science* **28**, 97-103.

Gonin, M., Quardokus, E. M., O'Donnol, D., Maddock, J. & Brun, Y. V. (2000). Regulation of stalk elongation by phosphate in *Caulobacter crescentus*. *Journal of Bacteriology* **182**, 337-347.

Gottlieb, D. (1976). Production and role of antibiotics in soil. *Journal of Antibiotics* **29**, 988-1000.

Haas, D., Blumer, C. & Keel, C. (2000). Biocontrol ability of fluorescent pseudomonads genetically dissected: importance of positive feedback regulation. *Current Opinion in Biotechnology* **11**, 290-297.

Hadar, Y., Harman, G. E., Taylor, A. G. & Norton, J. M. (1983). Effects of pregermination of pea and cucumber seeds and of seed treatment with *Enterobacter cloacae* on rots caused by *Pythium* spp. *Phytopathology* **73**, 1322-1325.

Hamdan, H., Weller, D. M. & Thomashow, L. S. (1991). Relative importance of fluorescent siderophores and other factors in biological control of *Gaeumannomyces graminis* var. *tritici* by *Pseudomonas fluorescens* 2-79 and M4-80R. *Applied and Environmental Microbiology* **57**, 3270-3277.

Hammer, P. E., Burd, W., Hill, D. S., Ligon, J. M. & van Pee, K. H. (1999). Conservation of the pyrrolnitrin biosynthetic gene cluster among six pyrrolnitrin-producing strains. *FEMS Microbiology Letters* **180**, 39-44.

Hammer, P. E., Hill, D. S., Lam, S. T., van Pee, K.-H. & Ligon, J. M. (1997). Four genes from *Pseudomonas fluorescens* that encode the biosynthesis of pyrrolnitrin. *Applied and Environmental Microbiology* **63**, 2147-2154.

Hamood, A. N., Colmer, J. A., Ochsner, U. A. & Vasil, M. L. (1996). Isolation and characterization of a *Pseudomonas aeruginosa* gene, *ptxR*, which positively regulates exotoxin A production. *Molecular Microbiology* **21**, 97-110.

Hanahan, D. (1983). Studies on transformation of *Escherichia coli* with plasmids. *Journal of Molecular Biology* **166**, 557-580.

Harris, S. J., Shih, Y. L., Bentley, S. D. & Salmond, G. P. C. (1998). The *hexA* gene of *Erwinia carotovora* encodes a LysR homologue and regulates motility and the expression of multiple virulence determinants. *Molecular Microbiology* **28**, 705-717.

Heeb, S., Itoh, Y., Nishijyo, T., Schnider, U., Keel, C., Wade, J., Walsh, U., O'Gara, F. & Haas, D. (2000). Small, stable shuttle vectors based on the minimal pVS1 replicon for use in Gram-negative, plant associated bacteria. *Molecular Plant Microbe Interactions* **13**, 232-237.

Hess, J. F., Bourret, R. B. & Simon, M. I. (1988a). Histidine phosphorylation and phosphoryl group transfer in bacterial chemotaxis. *Nature* **336**, 139-43.

Hess, J. F., Oosawa, K., Kaplan, N. & Simon, M. I. (1988b). Phosphorylation of three proteins in the signaling pathway of bacterial chemotaxis. *Cell* **53**, 79-87.

Higgins, C. F. (1992). ABC transporters: from microorganisms to man. *Annual review of Cell Biology* **8**, 67-113.

Hill, D. S., Stein, J. I., Torkiewitz, N. R., Morse, A. M., Howell, C. R., Pachlatko, J. P., Becker, J. O. & Ligon, J. M. (1994). Cloning of genes involved in the synthesis of pyrrolnitrin from *Pseudomonas fluorescens* and role of pyrrolnitrin synthesis in biological control of plant disease. *Applied and Environmental Microbiology* **60**, 78-85.

Holloway, B. W., Krishnapillai, V. & Morgan, A. F. (1977). Chromosomal genetics of *Pseudomonas*. *Microbial Reviews* **43**, 73-102.

Howell, C. R. & Stipanovic, R. D. (1979). Control of *Rhizoctonia solani* on cotton seedlings with *Pseudomonas fluorescens* and with an antibiotic produced by the bacterium. *Phytopathology* **69**, 480-482.

Howell, C. R. & Stipanovic, R. D. (1980). Suppression of *Pythium ultimum*-induced damping-off of cotton seedlings by *Pseudomonas fluorescens* and its antibiotic, pyoluteorin. *Phytopathology* **70**, 712-715.

Howie, W. J., Cook, R. J. & Weller, D. M. (1987). Effects of soil matric potential and cell motility on wheat root colonization by fluorescent pseudomonads suppressive to take-all. *Phytopathology* **77**, 286-292.

Howie, W. J. & Suslow, T. V. (1991). Role of antibiotic biosynthesis in the inhibition of *Pythium ultimum* in the cotton spermosphere and rhizosphere by *Pseudomonas fluorescens*. *Molecular Plant Microbe Interactions* **4**, 393-399.

Hrabak, E. M. & Willis, D. K. (1992). The *lemA* gene required for pathogenicity of *Pseudomonas syringae* pv. *syringae* on bean is a member of a family of two-component regulators. *Journal of Bacteriology* **174**, 3011-3020.

Huang, J., Denny, T. P. & Schell, M. A. (1993). VsrB, a regulator of virulence genes of *Pseudomonas solanacearum*, is homologous to sensors of the two-component regulator family. *Journal of Bacteriology* **175**, 6169-6178.

Huisman, G. W., Siegele, D. A., Zambrano, M. M. & Kolter, R. (1996). Morphological and physiological changes during stationary phase. In *Escherichia coli and Salmonella*, pp. 1672-1682. Edited by F. C. Neidhardt, R. Curtiss III, J. L. Ingraham, E. C. C. Lin, K. B. Low, B. Magasanik, W. S. Reznikoff, M. Riley, M. Schaechter & H. E. Umbarger. Washington D.C: ASM Press.

Igo, M. M., Ninfa, A. J. & Silhavy, T. J. (1989). A bacterial environmental sensor that functions as a protein kinase and stimulates transcriptional activation. *Genes and Development* **3**, 598-605.

Jacques, M. (1996). Role of lipo-oligosaccharides and lipopolysaccharides in bacterial adherence. *Trends in Microbiology* **4**, 408-410.

Jaeger, C. H., Lindow, S. E., Miller, S., Clark, E. & Firestone, M. K. (1999). Mapping of sugar and amino acid availability in soil around roots with bacterial sensors of sucrose and Tryptophan. *Applied and Environmental Microbiology* **65**, 2685-2690.

James, D. W. J. & Gutterson, N. I. (1986). Multiple antibiotics produced by *Pseudomonas fluorescens* HV37a and their differential regulation by glucose. *Applied and Environmental Microbiology* **52**, 1183-1189.

Jiang, W., Metcalf, W. W., Lee, K. S. & Wanner, B. L. (1995). Molecular cloning, mapping, and regulation of Pho regulon genes for phosphonate breakdown by the phosphonatase pathway of *Salmonella typhimurium* LT2. *Journal of Bacteriology* **177**, 6411-6421.

Jjemba, P. K. & Alexander, M. (1999). Possible determinants of rhizosphere competence of bacteria. *Soil Biology and Biochemistry* **31**, 623-632.

Jordan, S. R. & Pabo, C. O. (1988). Structure of the lambda complex at 2.5 A resolution: details of the repressor-operator interactions. *Science* **242**, 893-899.

Kearns, L. P. & Mahanty, H. K. (1998). Antibiotic production by *Erwinia herbicola* Eh1087: Its role in inhibition of *Erwinia amylovora* and partial characterization of antibiotic biosynthesis genes. *Applied and Environmental Microbiology* **64**, 1837-1844.

Keel, C., Voisard, C., Berling, C. H., Kahr, G. & Defago, G. (1989). Iron sufficiency, a prerequisite for the suppression of tobacco black root rot by *Pseudomonas fluorescens* strain CHA0 under gnotobiotic conditions. *Phytopathology* **79**, 584-589.

Keel, C., Weller, D. M., Natsch, A., Defago, G., Cook, R. J. & Thomashow, L. S. (1996). Conservation of the 2,4-diacetylphloroglucinol biosynthesis locus among fluorescent *Pseudomonas* strains from diverse geographic locations. *Applied and Environmental Microbiology* **62**, 552-563.

Keel, C., Wirthner, P. H., Oberhansli, T. H., Voisard, C., Burger, Haas, D. & Defago, G. (1990). Pseudomonads as antagonists of plant pathogens in the rhizosphere: role of the antibiotic 2,4-diacetylphloroglucinol in the suppression of black rot of tobacco. *Symbiosis* **9**, 327-341.

Kim, Y. C., Miller, C. D. & Anderson, A. J. (2000). Superoxide dismutase activity in *Pseudomonas putida* affects utilization of sugars and growth on root surfaces. *Applied and Environmental Microbiology* **66**, 1460-1467.

King, E. B. & Parke, J. L. (1993). Biocontrol of Aphanomyces root-rot and Pythium damping-off by *Pseudomonas cepacia* AMMD on four pea cultivars. *Plant Disease* **77**, 1185-1188.

King, E. O., Ward, M. K. & Raney, D. E. (1954). Two simple media for the demonstration of pyocyanin and fluorescen. *Journal of Laboratory and Clinical Medicine* **44**, 301-307.

Kirner, S., Hammer, P. E., Hill, D. S., Altmann, A., Fischer, I., Weislo, L. J. & Lanahan (1998). Functions encoded by pyrrolnitrin biosynthetic genes from *Pseudomonas fluorescens*. *Journal of Bacteriology* **180**, 1939-1943.

Kirner, S., Krauss, S., Sury, G., Lam, S. T., Ligon, J. M. & vanPee, K. H. (1996). The non-haem chloroperoxidase from *Pseudomonas fluorescens* and its relationship to pyrrolnitrin biosynthesis. *Microbiology* **142**, 2129-2135.

Kitten, T., Kinscherf, T. G., McEvoy, J. L. & Willis, D. K. (1998). A newly identified regulator is required for virulence and toxin production in *Pseudomonas syringae*. *Molecular Microbiology* **28**, 917-929.

Kleckner, N., Bender, J. & Gottesman, S. (1991). Uses of transposons with an emphasis on Tn*10*. *Methods in Enzymology* **204**, 139-180.

Kloepper, J. W., Leong, J., Teintze, M. & Schroth, M. N. (1980a). Enhanced plant growth by siderophores produced by plant growth-promoting rhizobacteria. *Nature* **286**, 885-886.

Kloepper, J. W., Leong, J., Teintze, M. & Schroth, M. N. (1980b). *Pseudomonas* siderophores: a mechanism explaining disease-suppressive soils. *Current Microbiology* **4**, 317-320.

Kohara, Y., Akiyama, K. & Isono, K. (1987). The physical map of the whole *E. coli* chromosome: application of a new strategy for rapid analysis and sorting of a large genomic library. *Cell* **50**, 495-508.

Kohli, R. M. & Massey, V. (1998). The oxidative half-reaction of old yellow enzyme. *The Journal of Biological Chemistry* **273**, 32763-32770.

Kolter, R., Siegele, D. A. & Tormo, A. (1993). The stationary phase of the bacterial life cycle. *Annual review of microbiology* **47**, 855-874.

Kowalczykowski, S. C., Dixon, D. A., Eggleston, A. K., Lauder, S. D. & Rehrauer, W. M. (1994). Biochemistry of homologous recombination in *Escherichia coli*. *Microbiological reviews* **58**, 401-465.

Kraus, J. & Loper, J. E. (1992). Lack of evidence for a role of antifungal metabolite production by *Pseudomonas fluorescens* Pf-5 in biological control of *Pythium* damping-off of cucumber. *Phytopathology* **82**, 264-271.

Kraus, J. & Loper, J. E. (1995). Characterisation of a genomic region required for the production of the antibiotic pyoluteorin by the biological control agent *Pseudomonas fluorescens* Pf-5. *Applied and Environmental Microbiology* **61**, 849-854.

Krummenacher, P. & Narberhaus, F. (2000). Two genes encoding a putative multidrug efflux pump of the RND/MFP family are cotranscribed with an *rpoH* gene in *Bradyrhizobium japonicum*. *Gene* **241**, 247-54.

Labbe, D., Garnon, J. & Lau, P. C. K. (1997). Characterization of the genes encoding a receptor-like histidine kinase and a cognate response regulator from a biphenyl/polychlorobiphenyl-degrading bacterium, *Rhodococcus* sp. strain M5. *Journal of Bacteriology* **179**, 2772-2776.

Lange, R. & Hengge-Aronis, R. (1991). Identification of a central regulator of stationary-phase gene expression in *Escherichia coli*. *Molecular Microbiology* **5**, 49-59.

Laurie, A. D. & Lloyd-Jones, G. (1999). The *phn* genes of *Burkholderia* sp. strain RP007 constitute a divergent gene cluster for polycyclic aromatic hydrocarbon catabolism. *Journal of Bacteriology* **181**, 531-540.

Laville, J., Blumer, C., Von Schroetter, C., Gaia, V., Defago, G., Keel, C. & Haas, D. (1998). Characterization of the *hcnABC* gene cluster encoding hydrogen cyanide synthase and anaerobic regulation by ANR in the strictly aerobic biocontrol agent *Pseudomonas fluorescens* CHA0. *Journal of Bacteriology* **180**, 3187-96.

Laville, J., Voisard, C., Keel, C., Maurhofer, M., Defago, G. & Haas, D. (1992). Global control in *Pseudomonas fluorescens* mediating antibiotic synthesis and suppression of black root rot of tobacco. *Proceedings Of The National Academy Of Sciences Of The United States Of America* **89**, 1562-1566.

Leeman, M., van Pelt, J. A., Den Ouden, F. M., Heinsbroek, M., Bakker, P. A. H. M. & Schippers, B. (1995a). Induction of systemic resistance against Fusarium wilt of radish by lipopolysaccharides of *Pseudomonas fluorescens*. *Phytopathology* **85**, 1021-1027.

Leeman, M., van Pelt, J. A., Hendrickx, M. J., Scheffer, R. J., Bakker, P. A. H. M. & Schippers, B. (1995b). Biocontrol of Fusarium wilt of radish in commercial greenhouse

trials by seed treatment with *Pseudomonas fluorescens* WCS374. *Phytopathology* **85**, 1301-1305.

Lefevre, P., Braibant, M., de Wit, L., Kalai, M., Roeper, D., Grotzinger, J., Delville, J. P., Peirs, P., Ooms, J., Huygen, K. & Content, J. (1997). Three different putative phosphate transport receptors are encoded by the *Mycobacterium tuberculosis* genome and are present at the surface of *Mycobacterium bovis* BCG. *Journal of Bacteriology* **179**, 2900-2906.

Leong, J. (1986). Siderophores: their biochemistry and possible role in the biocontrol of plant pathogens. *Annual review of Phytopathology* **24**, 187-209.

Lesse, A. J., Campagnari, A. A., Bittner, W. E. & Apicella, M. A. (1990). Increased resolution of lipopolysaccharides and lipooligosaccharides utilizing tricine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis. *Journal of Immunological methods* **126**, 109-117.

Levin, J. C. & Wessels, M. R. (1998). Identification of *csrR/csrS*, a genetic locus that regulates hyaluronic acid capsule synthesis in group A *Streptococcus*. *Molecular Microbiology* **30**, 209-219.

Liu, S. D. & Baker, R. (1980). Mechanism of Biological Control in Soil Suppressive to *Rhizoctonia solani*. *Phytopathology* **70**, 404-412.

Lodge, J., Busby, S. & Piddock, L. (1993). Investigation of the *Pseudomonas aeruginosa ampR* gene and its role at the chromosomal *ampC* beta-lactamase promoter. *FEMS Microbiology Letters* **111**, 315-319.

Loper, J. E. (1988). Role of fluorescent siderophore production in biological control of *Pythium ultimum* by a *Pseudomonas fluorescens* strain. *Phytopathology* **78**, 166-172.

Lucas, R. L., Lostroh, C. P., DiRusso, C. C., Spector, M. P., Wanner, B. L. & Lee, C. A. (2000). Multiple factors independently regulate *hilA* and invasion gene expression in *Salmonella enterica* serovar *typhimurium*. *Journal of Bacteriology* **182**, 1872-1882.

Luo, J., Burns, G. & Sokatch, J. R. (1993). Construction of chromosomal *recA* mutants of *Pseudomonas putida* PpG2. *Gene* **136**, 263-266.

Ma, D., Cook, D. N., Alberti, M., Pon, N. G., Nikaido, H. & Hearst, J. E. (1993). Molecular cloning and characterization of *acrA* and *acrE* genes of *Escherichia coli*. *Journal of Bacteriology* **175**, 6299-6313.

Magdalena, J., Gerard, C., Joris, B., Forsman, M. & Dusart, J. (1997). The two beta-lactamase genes of *Streptomyces cacaoi, blaL* and *blaU*, are under the control of the same regulatory system. *Molecular and General Genetics* **255**, 187-193.

Martin, J. F. & Demain, A. L. (1980). Control of antibiotic biosynthesis. *Microbiological Reviews* **44**, 230-251.

Maurhofer, M., Keel, C., Schnider, U., Voisard, C., Haas, D. & Defago, G. (1992). Influence of enhanced antibiotic production in *Pseudomonas fluorescens* strain CHA0 on its disease suppressive capacity. *Phytopathology* **82**, 190-195.

Mavrodi, D. V., Ksenzenko, V. N., Bonsall, R. F., Cook, R. J., Boronin, A. M. & Thomashow (1998). A seven-gene locus for synthesis of phenazine-1-carboxylic acid by *Pseudomonas fluorescens*. *Journal of Bacteriology* **180**, 2541-2548.

Mazzola, M., Cook, R. J., Thomashow, L. S., Weller, D. M. & Pierson, L. S. (1992). Contribution of phenazine antibiotic biosynthesis to the ecological competence of fluorescent pseudomonads in soil habitats. *Applied and Environmental Microbiology* **58**, 2616-2624.

Mazzola, M., Fujimoto, D. K., Thomashow, L. S. & Cook, R. J. (1995). Variation in sensitivity of *Gaeumannomyces graminis* to antibiotics produced by fluorescent *Pseudomonas* spp and effect on biological control of take-all of wheat. *Applied and Environmental Microbiology* **61**, 2554-2559.

McLoughlin, T. J., Quinn, J. P., Bettermann, A. & Bookland, R. (1992). *Pseudomonas cepacia* suppression of sunflower wilt fungus and role of antifungal compounds in controlling the disease. *Applied and Environmental Microbiology* **58**, 1760-1763.

Miller, J. H. (1972). *Experiments in molecular genetics*. Cold Spring Harbour, New York: Cold Spring Harbour Laboratory Press.

Miller, J. H. (1992). *A short course in bacterial genetics*. New York: Cold Spring Harbour Laboratory Press.

Miller, R. V. & Kokjohn, T. A. (1990). General microbiology of *recA*: environmental and evolutionary significance. *Annual review of microbiology* **44**, 365-394.

Mirleau, P., Delorme, S., Philippot, L., Meyer, J., Mazurier, S. & Lemanceau, P. (2000). Fitness in soil and rhizosphere of *Pseudomonas fluorescens* C7R12 compared with a C7R12 mutant affected in pyoverdine synthesis and uptake. *FEMS Microbiology Ecology* **34**, 35-44.

Monds, R. D. (2000). A molecular investigation of biofilm formation by *Pseudomonas aureofaciens* PA147-2. In *Department of Plant and Microbial Sciences*, pp. 137. Christchurch, New Zealand: University of Canterbury.

Morrison, D. A. (1966). Prodigiosin synthesis in mutants of *Serratia marcescens*. *Journal of Bacteriology* **91**, 1599-1604.

Natsch, A., Keel, C., Pfirter, H. A., Haas, D. & Defago, G. (1994). Contribution of the global regulator gene *gacA* to persistence and dissemination of *Pseudomonas fluorescens* biocontrol strain CHA0 introduced into soil microcosms. *Applied and Environmental Microbiology* **60**, 2553-2560.

Neilands, J. B. (1981). Microbial iron compounds. *Annual review of biochemistry* **50**, 715-731.

Newman, E. B., Lin, R. T. & D'ari, R. (1996). The leucine/Lrp regulon. In *Escherichia coli and Salmonella*, pp. 1513-1525. Edited by F. C. Neidhardt, R. Curtiss III, J. L. Ingraham, E. C. C. Lin, K. B. Low, B. Magasanik, W. S. Reznikoff, M. Riley, M. Schaechter & H. E. Umbarger. Washington D.C: ASM Press.

Nikata, T., Sakai, Y., Shibat, K., Kato, J., Kuroda, A. & Ohtake, H. (1996). Molecular analysis of the phosphate-specific transport (*pst*) operon of *Pseudomonas aeruginosa*. *Molecular and General Genetics* **250**, 692-8.

Novak, R., Cauwels, A., Charpentier, E. & Tuomanen, E. (1999). Identification of a *Streptococcus pneumoniae* gene locus encoding proteins of an ABC phosphate transporter and a two-component regulatory system. *Journal of Bacteriology* **181**, 1126-1133.

Nowak-Thompson, B., Chaney, N., Wing, J. S., Gould, S. J. & Loper, J. E. (1999). Characterization of the pyoluteorin biosynthetic gene cluster of *Pseudomonas fluorescens* Pf-5. *Journal of Bacteriology* **181**, 2166-2174.

Nowak-Thompson, B., Gould, S. J., Kraus, J. & Loper, J. E. (1994). Production of 2,4-diacetylphloroglucinol by the biocontrol agent *Pseudomonas fluorescens* Pf-5. *Canadian Journal of Microbiology* **40**, 1064-1066.

Nowak-Thompson, B., Gould, S. J. & Loper, J. E. (1997). Identification and sequence analysis of the genes encoding a polyketide synthase required for pyoluteorin biosynthesis in *Pseudomonas fluorescens* Pf-5. *Gene* **204**, 17-24.

Orvos, D. R., Lacy, G. H. & Cairns Jr, J. (1990). Genetically engineered *Erwinia carotovora*: survival, intraspecific competition, and effects upon selected bacterial genera. *Applied and Environmental Microbiology* **56**, 1689-1694.

O'Sullivan, D. J. & O'Gara, F. (1992). Traits of fluorescent *Pseudomonas* spp. involved in suppression of plant root pathogens. *Microbiological Reviews* **56**, 662-676.

O'Toole, G. A. & Kolter, R. (1998a). Flagellar and twitching motility are necessary for *Pseudomonas aeruginosa* biofilm development. *Molecular Microbiology* **30**, 295-304.

O'Toole, G. A. & Kolter, R. (1998b). Initiation of biofilm formation in *Pseudomonas fluorescens* WCS365 proceeds via multiple, convergent signalling pathways: a genetic analysis. *Molecular Microbiology* **28**, 449-461.

Parales, R. E. & Harwood, C. S. (1993). Construction and use of a new broad-host-range *lacZ* transcriptional fusion vector, pHRP309, for gram - bacteria. *Gene* **133**, 23-30.

Parke, D. (1996). Characterization of PcaQ, a LysR-type transcriptional activator required for catabolism of phenolic compounds, from *Agrobacterium tumefaciens*. *Journal of Bacteriology* **178**, 266-272.

Parkinson, J. S. & Kofoid, E. C. (1992). Communication modules in bacterial signaling proteins. *Annual review of genetics* **26**, 71-112.

Paulitz, T. C. & Loper, J. E. (1991). Lack of a role for fluorescent siderophore production in the biological control of *Pythium* damping-off of cucumber by a strain of *Pseudomonas putida*. *Phytopathology* **81**, 930-935.

Pierson, L. S., Keppenne, V. D. & Wood, D. W. (1994). Phenazine antibiotic biosynthesis in *Pseudomonas aureofaciens* 30-84 is regulated by PhzR in response to cell density. *Journal of Bacteriology* **176**, 3966-3974.

Pierson, L. S. & Pierson, E. A. (1996). Phenazine antibiotic production in *Pseudomonas aureofaciens*: Role in rhizosphere ecology and pathogen suppression. *FEMS Microbiology Letters* **136**, 101-108.

Pitcher, D., Saunders, N. & Owen, R. (1989). Rapid extraction of bacterial genomic DNA with guanidium thiocyanate. *Letters in Applied Microbiology* **8**, 151-156.

Prentki, P. & Krisch, H. M. (1984). *In vitro* insertional mutagenesis with a selectable DNA fragment. *Gene* **29**, 303-313.

Qi, Y., Kobayashi, Y. & Hulett, F. M. (1997). The *pst* operon of *Bacillus subtilis* has a phosphate-regulated promoter and is involved in phosphate transport but not in regulation of the pho regulon. *Journal of Bacteriology* **179**, 2534-2539.

Quandt, J. & Hynes, M. F. (1993). Versatile suicide vectors which allow direct selection for gene replacement in gram-negative bacteria. *Gene* **127**, 15-21.

Raaijmakers, J. M., Bonsall, R. F. & Weller, D. M. (1999). Effect of population density of *Pseudomonas fluorescens* on production of 2,4-diacetylphloroglucinol in the rhizosphere of wheat. *Phytopathology* **89**, 470-475.

Raaijmakers, J. M., Weller, D. M. & Thomashow, L. S. (1997). Frequency of antibiotic-producing *Pseudomonas* spp. in natural environments. *Applied and Environmental Microbiology* **63**, 881-887.

Rahme, L. G., Stevens, E. J., Wolfort, S. F., Shao, J., Tompkins, R. G. & Ausubel, F. M. (1995). Common virulence factors for bacterial pathogenicity in plants and animals. *Science* **268**, 1899-1902.

Rainey, P. & Travisano, M. (1998). Adaptive radiation in a heterogeneous environment. *Nature* **394**, 69-72.

Rainey, P. B. (1999). Adaptation of *Pseudomonas fluorescens* to the plant rhizosphere. *Environmental microbiology* **1**, 243-257.

Rainey, P. B. & Bailey, M. J. (1996). Physical and genetic map of the *Pseudomonas fluorescens* SBW25 chromosome. *Molecular Microbiology* **19**, 521-533.

Ramos, J. L., Duque, E., Godoy, P. & Segura, A. (1998). Efflux pumps involved in toluene tolerance in *Pseudomonas putida* DOT-T1E. *Journal of Bacteriology* **180**, 3323-3329.

Rankin, L. & Paulitz, T. C. (1994). Evaluation of rhizosphere bacteria for biological control of Pythium root-rot of greenhouse cucumbers in hydroponic culture. *Plant Disease* **78**, 447-451.

Rao, N. N. & Torriani, A. (1990). Molecular aspects of phosphate transport in *Escherichia coli*. *Molecular Microbiology* **4**, 1083-90.

Rhee, K. Y., Opel, M., Ito, E., Hung, S. P., Arfin, S. M. & Hatfield, G. W. (1999). Transcriptional coupling between the divergent promoters of a prototypic LysR-type regulatory system, the *ilvYC* operon of *Escherichia coli*. *Proceedings of the National Academy of Sciences of the United States of America* **96**, 14294-14299.

Robb, A. R. (1984). Physiology of asparagus (*Asparagus officinalis*) as related to the production of the crop. *N.Z. Journal of Experimental Agriculture* **12**, 251-260.

Ross, I. L., Alami, Y., Harvey, P. R., Achouak, W. & Ryder, M. H. (2000). Genetic diversity and biological control activity of novel species of closely related pseudomonads isolated from wheat field soils in South Australia. *Applied and Environmental Microbiology* **66**, 1609-1616.

Rothrock, C. S. & Gottlieb, D. (1984). Role of antibiosis in antagonism of *Streptomyces hygroscopicus* var. *geldanus* to *Rhizoctonia* in soil. *Canadian Journal of Microbiology* **30**, 1440-1447.

Ryder, M. H. & Rovira, A. D. (1993). Biological control of take-all of glasshouse-grown wheat using strains of *Pseudomonas corrugata* isolated from wheat field soil. *Soil Biology and Biochemistry* **25**, 311-320.

Ryder, M. H., Yan, Z., Terrace, T. E., Rovira, A. D., Tang, W. & Correll, R. L. (1999). Use of strains of *Bacillus* isolated in China to suppress take-all and *Rhizoctonia* root rot, and promote seedling growth of glasshouse-grown wheat in Australian soils. *Soil Biology and Biochemistry* **31**, 19-29.

Salles, B., Weisemann, J. M. & Weinstock, G. M. (1987). Temporal control of colicin E1 induction. *Journal of Bacteriology* **169**, 5028-5034.

Sambrook, J., Fritsch, E. F. & Maniatis, T. (1989). *Molecular cloning: a laboratory manual*, 2nd edn. New York: Cold Spring Harbor Laboratory Press.

Sancar, A., Hack, A. M. & Rupp, W. D. (1979). Simple method for identification of plasmid-coded proteins. *Journal of Bacteriology* **137**, 692-693.

Sanger, F., Nicklen, S. & Coulsen, A. R. (1977). DNA sequencing with chain-terminating inhibitors. *Proceedings of the National Academy of Sciences USA* **74**, 5463-5467.

Sano, Y. & Kageyama, M. (1987). The sequence and function of the *recA* gene and its protein in *Pseudomonas aeruginosa* PAO. *Molecular and General Genetics* **208**, 412-419.

Sarniguet, A., Kraus, J., Henkels, M. D., Muehlchen, A. M. & Loper, J. E. (1995). The sigma factor σ^S affects antibiotic production and biological control activity of *Pseudomonas fluorescens* Pf-5. *Proceedings of the National Academy of Sciences USA* **92**, 12255-12259.

Scheer, D. F. & Ellison, J. H. (1960). Asparagus performance as related to seedling vigor. *American Society for Horticultural Science* **76**, 370-375.

Schell, M. A. (1993). Molecular biology of the LysR family of transcriptional regulators. *Annual review of microbiology* **47**, 597-626.

Schell, M. A. (2000). Control of virulence and pathogenicity genes of *Ralstonia solanacearum* by an elaborate sensory network. *Annual review of Phytopathology* **38**, 263-292.

Scher, F. M. & Baker, R. (1980). Mechanism of biological control in a *Fusarium* suppressive soil. *Phytopathology* **70**, 412-417.

Scher, F. M., Kloepper, J. W., Singleton, C., Zaleska, I. & Lalberte, M. (1988). Colonization of soybean roots by *Pseudomonas* and *Serratia* species: relationship to bacterial motility, chemotaxis, and generation time. *Phytopathology* **78**, 1055-1059.

Schippers, B., Bakker, A. W. & Bakker, P. A. H. M. (1987). Interactions of deleterious and beneficial rhizosphere microorganisms and the effect of cropping practices. *Annual review of Phytopathology* **25**, 339-358.

Schnaitman, C. A. & Klena, J. D. (1993). Genetics of lipopolysaccharide biosynthesis in enteric bacteria. *Microbiological Reviews* **57**, 655-682.

Schnider-Keel, U., Seematter, A., Maurhofer, M., Blumer, C., Duffy, B., Gigot-Bonnefoy, C., Reimmann, C., Notz, R., Defago, G., Haas, D. & Keel, C. (2000). Autoinduction of 2,4-diacetylphloroglucinol biosynthesis in the biocontrol agent *Pseudomonas fluorescens* CHA0 and repression by the bacterial metabolites salicylate and pyoluteorin. *Journal of Bacteriology* **182**, 1215-1225.

Schroth, M. N. & Hancock, J. G. (1982). Disease suppressive soil and root colonizing bacteria. *Science* **216**, 1376-1381.

Silby, M. W. & Mahanty, H. K. (2000). The importance of *recA* mutant strains for the study of antifungal genes in *Pseudomonas aureofaciens* PA147-2. *Canadian Journal of Microbiology* **46**, 744-748.

Silhavy, T., Berman, M. & Enquist, L. (1984). *Experiments with gene fusions*. Cold Spring Harbour, NY: Cold Spring Harbour Laboratory Press.

Simon, R., Priefer, U. & Puhler, A. (1983). A broad host range mobilisation system for *in vivo* engineering: Transposon mutagenesis in gram-negative bacteria. *Biotechnology* **1**, 748-751.

Slininger, P. J. & Jackson, M. A. (1992). Nutritional factors regulating growth and accumulation of phenazine 1-carboxylic acid by *Pseudomonas fluorescens* 2-79. *Applied Microbiology and Biotechnology* **37**, 388-392.

Smith, J. J., Offord, L. C., Holderness, M. & Saddler, G. S. (1995). Genetic diversity of *Burkholderia solanacearum* (synonym *Pseudomonas solanacearum*) race 3 in Kenya. *Applied and Environmental Microbiology* **61**, 4263-4268.

Staskawicz, B., Dahlbeck, D., Keen, N. & Napoli, C. (1987). Molecular characterization of cloned avirulence genes from Race 0 and Race 1 of *Pseudomonas syringae* pv. *glycinea*. *Journal of Bacteriology* **169**, 5789-5794.

Steed, P. M. & Wanner, B. L. (1993). Use of the rep technique for allele replacement to construct mutants with deletions of the *pstSCAB-phoU* operon: evidence of a new role for the PhoU protein in the phosphate regulon. *Journal of Bacteriology* **175**, 6797-6809.

Steel, R. G. D. & Torrie, J. H. (1976). *Introduction to Statistics*. New York: McGraw-Hill.

Stephens, P. M., O'Sullivan, M. & O'Gara, F. (1987). Effect of bacteriophage on colonization of sugarbeet roots by fluorescent *Pseudomonas* spp. *Applied and Environmental Microbiology* **53**, 1164-1167.

Stock, J. B., Stock, A. M. & Mottonen, J. M. (1990). Signal transduction in bacteria. *Nature* **344**, 395-400.

Stutz, E., Kahr, G. & Defago, G. (1989). Clays involved in suppression of tobacco black root rot by a strain of *Pseudomonas fluorescens*. *Soil Biology and Biochemistry* **21**, 361-366.

Suslow, T. V. & Schroth, M. N. (1982). Rhizobacteria of sugar beets: effects of seed application and root colonization on yield. *Phytopathology* **72**, 199-206.

Takemaru, K., Mizuno, M. & Kobayashi, Y. (1996). A *Bacillus subtilis* gene cluster similar to the *Escherichia coli* phosphate-specific transport (*pst*) operon: evidence for a tandemly arranged *pstB* gene. *Microbiology* **142**, 2017-2020.

Tari, P. H. & Anderson, A. J. (1988). Fusarium wilt suppression and agglutinability of *Pseudomonas putida*. *Applied and Environmental Microbiology* **54**, 2037-2041.

Thomashow, L. S. & Weller, D. M. (1988). Role of a phenazine antibiotic from *Pseudomonas fluorescens* in biological control of *Gaeumannomyces graminis* var. *tritici*. *Journal of Bacteriology* **170**, 3499-508.

Thomashow, L. S., Weller, D. M., Bonsall, R. F. & Pierson, L. S. (1990). Production of the antibiotic phenazine-1-carboxylic acid by fluorescent *Pseudomonas* species in the rhizosphere of wheat. *Applied and Environmental Microbiology* **56**, 908-912.

Toman, Z., Dambly-Chaudiere, C., Tenenbaum, L. & Radman, M. (1985). A system for detection of genetic and epigenetic alterations in *Escherichia coli* induced by DNA-damaging agents. *Journal of Molecular Biology* **186**, 97-105.

Turner, J. M. & Messenger, A. J. (1986). Occurence, biochemistry and physiology of phenazine pigment production. *Advances in Microbial Physiology* **27**, 211-275.

van Peer, R., Niemann, G. J. & Schippers, B. (1991). Induced resistance and phytoalexin accumulation in biological control of fusarium wilt of carnation by *Pseudomonas* sp. strain WCS417r. *Phytopathology* **81**, 728-734.

Velasco, A., Alonso, S., Garcia, J. L., Perera, J. & Daiz, E. (1998). Genetic and functional analysis of the styrene catabolic cluster of *Pseudomonas* sp. strain Y2. *Journal of Bacteriology* **180**, 1063-1071.

Voisard, C., Keel, C., Haas, D. & Defago, G. (1989). Cyanide production by *Pseudomonas fluorescens* helps auppress black root-rot of tobacco under gnotobiotic conditions. *Embo Journal* **8**, 351-358.

Wandersman, C. & Letoffe, S. (1993). Involvement of lipopolysaccharide in the secretion of *Escherichia coli* α-haemolysin and *Erwinia chrysanthemi* proteases. *Molecular Microbiology* **7**, 141-150.

Wanner, B. L. (1995). Signal transduction and cross regulation in the *Escherichia coli* phosphate regulon by PhoR, CreC, and acetyl phosphate. In *Two-component signal transduction*, pp. 203-221. Edited by J. A. Hoch & T. J. Silhavy. Washington, DC: American Society for Microbiology.

Wanner, B. L. (1996). Phosphorus assimilation and control of the phosphate regulon. In *Escherichia coli and Salmonella*, pp. 1357-1381. Edited by F. C. Neidhardt, R. Curtiss III, J. L. Ingraham, E. C. C. Lin, K. B. Low, B. Magasanik, W. S. Reznikoff, M. Riley, M. Schaechter & H. E. Umbarger. Washington D.C: ASM Press.

Wanner, B. L. & Wilmes-Riesenberg, M. R. (1992). Involvement of phosphotransacetylase, acetate kinase, and acetyl phosphate synthesis in control of the phosphate regulon in *Escherichia coli*. *Journal of Bacteriology* **174**, 2124-2130.

Wei, G., Kloepper, J. W. & Tuzun, S. (1991). Induction of systemic resistance of cucumber to *Colletotrichum orbiculare* by select strains of plant growth-promoting rhizobacteria. *Phytopathology* **81**, 1508-1512.

Weller, D. M. (1988). Biological control of soilborne plant pathogens in the rhizosphere with bacteria. *Annual review of Phytopathology* **26**, 379-407.

Williams, S. T. & Vickers, J. C. (1986). The ecology of antibiotic production. *Microbial Ecology* **12**, 43-52.

Willis, D. K., Hrabak, E. M., Lindow, S. & Panopoulos, N. J. (1988). Construction and characterisation of *Pseudomonas syringae recA* mutant strains. *Molecular plant-microbe interactions* **1**, 80-86.

Winkler, H. H. (1966). A hexose-phosphate transport system in *Escherichia coli*. *Biochemica et biophysica acta* **117**, 231-240.

Wood, D. W. & Pierson, L. S., III (1996). The *phzI* gene of *Pseudomonas aureofaciens* 30-84 is responsible for the production of a diffusible signal required for phenazine antibiotic production. *Gene* **168**, 49-53.

Woods, W. G. & Dyall-Smith, M. L. (1997). Construction and analysis of a recombination-deficient (*radA*) mutant of *Haloferax volcanii*. *Molecular Microbiology* **23**, 791-797.

Wu, H., Kosaka, H., Kato, J., Kuroda, A., Ikeda, T., Takiguchi, N. & Ohtake, H. (1999). Cloning and characterisation of *Pseudomonas putida* genes encoding the phosphate-specific transport system. *Journal of Bioscience and Bioengineering* **87**, 273-279.

Yanisch-Perron, C., Vieira, C. & Messing, J. (1985). Improved M13 phage cloning vectors and host strains: nucleotide sequence of M13mp18 and pUC19 vectors. *Gene* **33**, 103-119.

Zambrano, M. M. & Kolter, R. (1996). GASPing for life in stationary phase. *Cell* **86**, 181-184.

Zambrano, M. M., Siegele, D. A., Almiron, M., Tormo, A. & Kolter, R. (1993). Microbial competition: *Escherichia coli* mutants that take over stationary phase cultures. *Science* **259**, 1757-60.

Zinser, E. R. & Kolter, R. (1999). Mutations enhancing amino acid catabolism confer a growth advantage in stationary phase. *Journal of Bacteriology* **181**, 5800-5807.

Zinser, E. R. & Kolter, R. (2000). Prolonged stationary-phase incubation selects for *lrp* mutations in *Escherichia coli* K-12. *Journal of Bacteriology* **182**, 4361-4365.

Appendix 1

Media, Buffers, Solutions

Media

H-Top Agar

1%	Bactotryptone
0.8%	NaCl
0.8%	Agar

In dH_2O; Autoclave

King's medium B (King et al., 1954)

2% w/v	bactotryptone
65mM	K_2HPO_4 anhydrous
60mM	$MgSO_4.7H_2O$
1.5% w/v	Glycerol

In dH_2O; Autoclaved

K10 medium

A 1:10 dilution of King's B

K10P medium

10% strength King's B prepared without the K_2HPO_4
A number after K10P- signifies the amount of added inorganic phosphate

M9 minimal medium (Sambrook et al., 1989)

47.7M	$Na_2HPO_4.7H_2O$
22mM	KH_2PO_4
8.5mM	NaCl
18.7	NH_4Cl

In dH_2O; Autoclave, then add desired carbon source at appropriate concentration

Minimal 'A' medium (Miller, 1972)

60mM	K_2HPO_4
33mM	KH_2PO_4
7.6mM	$(NH_4)_2SO_4$
1.7mM	Sodium citrate.$2H_2O$

In dH_2O; Autoclave, then add desired carbon source at appropriate concentration

Luria Bertani Medium (LB)

1% w/v Bactotryptone
0.5% w/v Yeast extract
0.5% w/v NaCl
In dH_2O; Autoclaved

Solidify by adding 1.5% agar prior to autoclaving.

LBMM (for miniTn*10* mutagenesis)

LB broth supplemented with 0.2% maltose and 10mM $MgSO_4$ (after autoclaving).

PGTIPS medium

25mM KCl
8mM $(NH_4)_2SO_4$
1.2mM $MgSO_4$
20mM Tris-HCl (pH7)
0.2% w/v Glycerol
In dH_2O. Autoclaved.

Pseudomonas minimal medium (Kirner et al., 1996)

35mM $K_2HPO_4.3H_2O$
22mM KH_2PO_4
8mM $(NH_4)_2SO_4$
25mM Sodium succinate
In dH_2O; Autoclaved
1.2mM $MgSO_4$ (added after autoclaving)

Solidify by adding 1.5% w/v agar prior to autoclaving

SOC medium (for electroporation) (Sambrook et al., 1989)

2% w/v bactotryptone
0.5% w/v Yeast extract
10mM NaCl
2.5mM KCl
10mM $MgCl_2$
10mM $MgSO_4$
0.36% w/v Glucose
In dH_2O; Autoclaved

T medium (Nikata et al., 1996)

80mM	TrisHCl pH7.6
85mM	NaCl
20mM	KCl
20mM	NH_4Cl
2.5mM	Na_2SO_4
1mM	$MgCl_2$
3μM	$FeCl_3$
0.2% w/v	Glucose

In dH_2O; Autoclaved

Inorganic phosphate is added to this medium in defined quantities. A subscript number indicates the addition of that concentration of inorganic phosphate (in mM).

Minimal bioassay medium

T medium supplemented with the desired inorganic phosphate concentration and 2% glucose (w/v).

Tris Buffered Potato dextrose agar (TPDA)

PDA is made up as described by the manufacturer (Oxoid), except TrisHCl (pH7.6) is included at a final concentration of 80mM.

Buffers and solutions

All buffers and solutions were made using dH_2O.

Common buffers and solutions

$T_{10}E_1$ (TE)

10mM	Tris-HCl pH8.0
1mM	EDTA pH8.0

50x TAE

242g	Tris
100mL	0.5M EDTA (pH8.0)
57.1mL	Glacial acetic acid

dH_2O to 1 litre; pH adjusted to 8.0; Dilute to 1x for use.

10x TBE

121g	Tris
51.35g	Boric acid
3.72g	EDTA $Na_2 2H_2O$

dH_2O to one litre; pH adjusted to 8.0; Dilute to 0.5x for use

20x SSC

175.3g	NaCl
88.2g	Na_3Citrate

dH_2O to 1 litre; pH7 (adjust with HCl).

Loading buffer for agarose gel electrophoresis

30%	Glycerol
0.25%	Bromophenol blue
0.25%	Xylene cyanol

RNaseA to final concentration of 10µg/mL

GES lysis solution for genomic DNA preparation

5M	Guanidium thiocyanate
100mM	EDTA
0.5%	Sarkosyl

Dissolve 60g guanidium thiocyanate in 20mL 0.5M EDTA and 20mL dH_2O with heating (65°C). Add 5mL 10% sarkosyl, make volume to 100mL (dH_2O), filter through 0.45µm filter

Solutions for alkaline lysis plasmid DNA preparations (small and large scale)

Solution 1

1%	Glucose
25mM	Tris-HCl pH8.0
10mM	EDTA

Solution 2

0.2M	NaOH
1%	SDS

Solution 3

3M	Sodium acetate pH4.8

5M LiCl

21.2g	LiCl

dH_2O to 100mL

β-galactosidase assay solutions

Z Buffer

60mM	$Na_2HPO_4.7H_2O$
40mM	$NaH_2PO_4.H_2O$
10mM	KCl
1mM	$MgSO_4.7H_2O$
50mM	β-mercaptoethanol

pH7; Do not autoclave

1M Na_2CO_3

10.6g	Na_2CO_3

dH_2O to 100mL; do not autoclave

SDS-PAGE solutions and gel recipes

Resolving gel buffer

1.5M	Tris-HCl pH8.8 (adjusted with HCl)

Stacking gel buffer

0.5M	Tris-HCl pH6.8 (adjusted with HCl)

Tank buffer

25mM	Tris
192mM	Glycine
0.1%	SDS

2x Sample treatment buffer

2.5mL	0.5M Tris-HCl
4.0mL	10% SDS
2.0mL	Glycerol
1.0mL	2-mercaptoethanol
0.2mL	0.1% w/v bromophenol blue

dH_2O to 10mL; pH6.8

Coomassie stain stock

1%	Coomassie brilliant blue R-250

Stir to dissolve; Filter through Whatman number 3.

Coomassie brilliant blue R-250 stain solution for protein gels

62.5mL	Coomassie stain stock
250mL	Methanol
50mL	Acetic acid

dH_2O to 500mL.

Destain solution 1

500mL Methanol
100mL Acetic acid
dH_2O to 1 litre.

Destain solution 2

50mL Methanol
70mL Acetic acid
dH_2O to 1 litre.

SDS-PAGE protein gel recipes

	Separating gel[a]			Stacking gel
	8%	12%	16%	4%
Acrylamide/Bis[b]	26.67mL	40mL	53.3mL	1.3mL
Separating gel buffer	25mL	25mL	25ml	-
Stacking gel buffer	-	-	-	2.5mL
10% SDS	1mL	1mL	1mL	100µL
dH_2O	46.78mL	33.5mL	20.12mL	6.1mL
10% APS[c]	500µL	500µL	500µL	50µL
TEMED[c]	50µL	50µL	50µL	10µL
Total volume[d]	**100mL**	**100mL**	**100mL**	**10mL**

[a]Any combination can be used to pour gradient gels. [b]30% stock. [c]Add after 10 minutes degassing, and immediately prior to pouring the gel. [b]Can be scaled up or down to get desired volumes

Buffers and solutions for transfer of proteins to PVDF membrane

Transfer buffer

25mM Tris
192mM Glycine
20% v/v methanol

Coomassie brilliant blue G-250 stain stock

1% Coomassie brilliant blue G-250 in dH_2O
Stir and filter

PVDF membrane staining solution

1mL Coomassie G-250 stock
4mL Methanol
100µL Acetic acid
Make up to 10mL with dH_2O. Prepare fresh each time.

PVDF destain solution

50%	Methanol in dH_2O

Solutions and media for maxicell experiments

K medium

M9 medium with:

1%	Casamino acids
0.1µg/mL	Thiamine

Hershey salts (without sulphate) per litre

5.4g	NaCl
3g	KCl
1.1g	NH_4Cl
15mg	$CaCl_2.2H_2O$
200mg	$MgCl_2.6H_2O$
0.2mg	$FeCl_3.6H_2O$
87mg	KH_2PO_4
12.1g	Tris

In dH_2O. Adjust pH to 7.4 with HCl. Autoclave.

Sulphate free Hershey medium (add per 100mL Hershey salts)

2mL	Glucose
0.5mL	Threonine
1mL	Leucine
1mL	Proline
1mL	Arginine
0.1mL	Thiamine

Solutions for FIGE

0.5x TBE (as recommended by Biorad)

45mM	Tris
45mM	Borate
1.0mM	EDTA

In dH_2O; pH8.3

SE buffer

20mM	NaCl
50mM	EDTA

Lysis buffer

0.5M	EDTA
0.5mg/mL	Pronase E
1%	N-lauryl sarcosine

MC buffer for P1 transduction

100mM	$MgSO_4$
5mM	$CaCl_2$

Appendix 2

DNA sequencing, FIGE, and PCR parameters

LI-COR sequencing

Cycle in PCR machine

1. 95 2min
2. 95 30 sec
3. 50 for 15 sec
4. 70 for 15 sec
5. go to 2 29 times

Scanner control parameters

Data collection configuration file	66-cm-STD.col
Quick SequencIR configuration file	Quik66cm.col
Voltage	2000 Volts
Current	25.0mA
Power	45.0 Watts
Temperature	45.0°C
Scan speed	2 (= 1.2 frames/hour)
Prerun time	1 hour
Frames to collect	32

FIGE parameters

Voltage	Forward	180V
	Reverse	180V
Run time	10 hours	
Switch time	Initial fwd	1.2 seconds
	Final fwd	9 seconds
	Initial rev	0.4 seconds
	Final rev	3 seconds
Ramp	Linear	

RT-PCR cycle (finR expression analysis)

(Primers finexfwd and finexrev)

42°C 30 minutes (RT step)

95°C 5 minutes (one cycle)

94°C 30 seconds, 47°C 30 seconds, 72°C 90 seconds (35 cycles)

94°C 30 seconds, 47°C 30 seconds, 72°C 3 minutes (1 cycle)

PCR cycles

Amplification of pDEL1 insert

(Primers *finR*dfwd1 and *finR*drev1a)

95°C 5 minutes (one cycle)

94°C 30 seconds, 47°C 30 seconds, 72°C 90 seconds (30 cycles)

94°C 30 seconds, 47°C 30 seconds, 72°C 3 minutes (1 cycle)

Amplification of pDEL2 insert

(Primers *finR*dfwd2 and *finR*drev2)

95°C 5 minutes (one cycle)

94°C 30 seconds, 43°C 30 seconds, 72°C 90 seconds (30 cycles)

94°C 30 seconds, 43°C 30 seconds, 72°C 3 minutes (1 cycle)

Amplification of pDEL3 insert

(Primers *finR*dfwd 1 and *finR*drev2)

94°C 1 minutes (one cycle)

94°C 30 seconds, 55°C 30 seconds, 72°C 2 minutes (30 cycles)

94°C 30 seconds, 55°C 30 seconds, 72°C 3 minutes (1 cycle)

Appendix 3

Primers for Sequencing and PCR

Standard primers

IS*10*	5′ GGGATCATATGACAAGATGT 3′
IS*50*	5′ GCACGATGAAGAGCAGAAG 3′
T7 promoter	5′ GTAATACGACTCACTATAGGGC 3′
T3 promoter	5′ AATTAACCCTCACTAAAGGG 3′

Custom primers

Primers for RT-PCR

finexFwd	5′ AAGGAGTCATATGGACAAGC 3′
finexRev	5′ CCACGGGATCCGATCAAAGC 3′

Primers for construction of finR deletion

*finR*dfwd1	5′ CTGATCGCGAATAACCGATC 3′
*finR*dfwd2	5′ TCCATAAGGATCCTTAAACG 3′
*finR*drev1a	5′ TGGCACTGGATCCTGACTGG 3′
*finR*drev2	5′ AGGCACACCGCTGTAACTTC 3′

Primers for sequencing

PR138a	5′ CACTCAGAATTTCCCGAGCC 3′
REDSEQ	5′ ATCATCACAGAGGCTTCTCA 3′
*finT*fwd1	5′ ACTGGCTGGATGTGCTCGAC 3′
*finT*fwd2	5′ ACGACATCCTCGACTTCTCG 3′
*finT*rev1	5′ AACGGAATGTGCTCAAGCTC 3′

Appendix 4

DNA sequences

A4.1 Complete open reading frames

recA

a) Open reading frame

```
   1  ATGGACGACA ACAAGAAGAA AGCCTTGGCT GCGGCCATGG GACAGATCGA
  51  ACGTCAATTC GGCAAGGGTG CCGTAATGCG TATGGGCGAT CACGACCGTC
 101  AGGCGATCCC GGCTATCTCC ACTGGCTCTC TGGGTCTGGA CATCGCGCTC
 151  GGCATTGGCG GCCTGCCAAA AGGCCGTATC GTTGAGATCT ACGGTCCTGA
 201  ATCTTCCGGT AAAACCACCC TGACCCTGTC GGTGATTGCC CAGGCACAAA
 251  AAATGGGCGC CACCTGTGCG TTCGTCGATG CCGAGCACGC CCTGGATCCT
 301  AAATACACCG GCACGCTGGG CGTCAACGCT GACGACCTGC TGGTTTCGCA
 351  ACCGGACACC GGTGAGCAAG CGCTGGAAAT CACCGACATG CTGGTGCGCT
 401  CCAACGCCAT CGACGTGATC GTGGTCGACT CCGTGGCAGC CCTGGTACCC
 451  AAGGCTGAAA TCGAAGGCGA AATGGGCGAC ATGCACGTGG GCCTTCAAGC
 501  CCGCTTGATG TCCCAGGCCC TGCGTAAAAT CACCGGTAAC ATCAAGAACG
 551  CCAACTGCCT GGTGATCTTC ATCAACCAGA TCCGGATGAA GATCGGCGTG
 601  ATGTTCGGCA GCCCGGAAAC CACCACCGGT GGTAACGCGC TGAAGTTCTA
 651  CGCTTCGGTC CGTCTGGACA TCCGTCGTAC TGGCGCGGTG AAGGAAGGTG
 701  ACGAGGTTGT CGGTAGCGAA ACCCGCGTTA AAGTCGTGAA GAACAAGGTG
 751  GCCCCGCCGT TCCGTCAGGC CGAGTTCCAG ATTCTCTACG GCAAGGGTAT
 801  CTACCTGAAC GGCGAGATGA TCGACCTGGG CGTGCTGCAC GGTTTCGTCG
 851  AGAAATCCGG TGCCTGGTAT GCCTACAACG GCAGCAAGAT CGGCCAGGGC
 901  AAGGCCAACT CGGACAAGTT CCTTGCGGAT AACCCGGATG TTGCCGCTAC
 951  GCTCGAGAAG CAGATTCGCG ACAAGTTGCT GACCCCGGCA CCTGACGTCA
1001  ACGCTGCCGC CAACCGCGAG CCGGTTGAAG AAGTAGAAGA AGTCGACACT
1051  GACATCTGA
```

b) Highest Blast hits

```
                                                                  Score    E
Sequences producing significant alignments:                       (bits) Value

gi|151529|gb|M96558.1|PSERECAOE  Pseudomonas fluorescens (cl...  1608  0.0
gi|6689064|emb|AJ249265.1|PTO249265  Pseudomonas tolaasii re...  1600  0.0
gi|294374|gb|L12684.1|PSERECAO  Pseudomonas putida recA prot...   894  0.0
gi|1617591|gb|U70864.1|PPU70864  Pseudomonas putida RecA and...   886  0.0
gi|45383|emb|X52261.1|PAP7RECA  Pseudomonas aeruginosa PAM 7...   866  0.0
gi|15595198|ref|NC_002516.1|  Pseudomonas aeruginosa, comple...   850  0.0
gi|9949772|gb|AE004782.1|AE004782  Pseudomonas aeruginosa PA...   850  0.0
gi|45413|emb|X05691.1|PARECA  Pseudomonas aeruginosa recA gene    850  0.0
gi|286185|dbj|D13090.1|PSERESA2  Pseudomonas aeruginosa gene...   850  0.0
gi|248704|gb|S96898.1|S96898  recA [Azotobacter vinelandii, ...   690  0.0
```

c) Alignment with *P. fluorescens recA*

```
 Identities = 997/1059 (94%)
PA147: 1    atggacgacaacaagaagaaagccttggctgcggccatgggacagatcgaacgtcaattc 60
            ||||||||||||||||||||||||||||||||||| |||| ||||||||||||||||||
P.Flu: 156  atggacgacaacaagaagaaagccttggctgcggccctgggtcagatcgaacgtcaattc 215


PA147: 61   ggcaagggtgccgtaatgcgtatgggcgatcacgaccgtcaggcgatcccggctatctcc 120
            |||||||||||||||||||||||||||||||||||||||||| |||||||||||||| |||
P.flu: 216  ggcaagggtgccgtaatgcgtatgggcgatcacgaccgtcaagcgatcccggctatttcc 275
```

```
PA147: 121  actggctctctgggtctggacatcgcgctcggcattggcggcctgccaaaaggccgtatc 180
            ||||||||||||||||||||||||||| ||||||||||||||||||||||||||||||||
P.flu: 276  actggctctctgggtctggacatcgcactcggcattggcggcctgccaaaaggccgtatc 335

PA147: 181  gttgagatctacggtcctgaatcttccggtaaaaccaccctgaccctgtcggtgattgcc 240
            |||||| ||||||||||||||||||||||||||||||||||||||||||||||||||||||
P.flu: 336  gttgaaatctacggtcctgaatcttccggtaaaaccaccctgaccctgtcggtgattgcc 395

PA147: 241  caggcacaaaaaatgggcgccacctgtgcgttcgtcgatgccgagcacgccctggatcct 300
            || || |||||||||||||||||||||||||||||||||| ||||||||||||||| || ||
P.flu: 396  caagcgcaaaaaatgggcgccacctgtgcgttcgtcgacgccgagcacgcccttgacccg 455

PA147: 301  aaatacaccggcacgctgggcgtcaacgctgacgacctgctggtttcgcaaccggacacc 360
             ||||| |||| | |||||||||||||| ||||||||||||||||| || |||||||||
P.flu: 456  gaatacgccggtaagctgggcgtcaacgttgacgacctgctggtttcccagccggacacc 515

PA147: 361  ggtgagcaagcgctggaaatcaccgacatgctggtgcgctccaacgccatcgacgtgatc 420
            |||||||||||| ||||||||||||||||||||||||||||||||||||||||||||||||
P.flu: 516  ggtgagcaagccctggaaatcaccgacatgctggtgcgctccaacgccatcgacgtgatc 575

PA147: 421  gtggtcgactccgtggcagccctggtacccaaggctgaaatcgaaggcgaaatgggcgac 480
            |||||||||||||||| |||||||||| || ||||||||||||||||||||||||||||
P.flu: 576  gtggtcgactccgtggctgccctggtaccgaaagctgaaatcgaaggcgaaatgggcgac 635

PA147: 481  atgcacgtgggccttcaagcccgcttgatgtcccaggccctgcgtaaaatcaccggtaac 540
            |||||||||||||| ||||||||| |||||||||||| |||||||||| |||||||||
P.flu: 636  atgcacgtgggcctgcaagcccgcctgatgtcccaggcgctgcgtaaaattaccggtaac 695

PA147: 541  atcaagaacgccaactgcctggtgatcttcatcaaccagatccggatgaagatcggcgtg 600
            ||||||||||||||||||||||||||||||||||||||||||||| ||||||||||||||
P.flu: 696  atcaagaacgccaactgcctggtgatcttcatcaaccagatccgtatgaagatcggcgta 755

PA147: 601  atgttcggcagcccggaaaccaccaccggtggtaacgcgctgaagttctacgcttcggtc 660
            |||||||||||||||||||||| ||||||||||||||||||||||||||||||||||||
P.flu: 756  atgttcggcagcccggaaaccactaccggtggtaacgcgctgaagttctacgcttcggtc 815

PA147: 661  cgtctggacatccgtcgtactggcgcggtgaaggaaggtgacgaggttgtcggtagcgaa 720
            |||||||||||||| ||||| |||||||||||||||||||||| ||||| |||||||||
P.flu: 816  cgtctggacatccgccgtaccggcgcggtgaaggaaggtgacgaagttgttggtagcgaa 875

PA147: 721  acccgcgttaaagtcgtgaagaacaaggtggccccgccgttccgtcaggccgagttccag 780
            || |||||||||||||||||||||||| || ||||| |||||||||||| |||||||||
P.flu: 876  actcgcgttaaagtcgtgaagaacaaggtcgctccgcctttccgtcaggcagagttccag 935

PA147: 781  attctctacggcaagggtatctacctgaacggcgagatgatcgacctgggcgtgctgcac 840
            |||||||||||||||||||||||||||||||||||||||||||||| |||||||||||||
P.flu: 936  attctctacggcaagggtatctacctgaacggcgagatgattgacctgggcgtactgcac 995

PA147: 841  ggtttcgtcgagaaatccggtgcctggtatgcctacaacggcagcaagatcggccagggc 900
            |||||||||||||| ||||||||||||||||||||||||||||||||||||| ||||||
P.flu: 996  ggtttcgtcgagaagtccggtgcctggtatgcctacaacggcagcaagatcggtcagggc 1055

PA147: 901  aaggccaactcggacaagttccttgcggataacccggatgttgccgctacgctcgagaag 960
            |||||||||||||| ||||||||| || || ||||||||| | | ||| ||||| ||||||
P.flu: 1056 aaggccaactcggccaagttcctggcagacaacccggatatcgtcgccacgcttgagaag 1115

PA147: 961  cagattcgcgacaagttgctgaccccggcacctgacgtcaacgctgccgccaaccgcgag 1020
            |||||||||||||||| |||||||||| || || ||||| || ||||||||||||||||||
P.flu: 1116 cagattcgcgacaagctgctgaccccagcgccagacgtgaaagctgccgccaaccgcgag 1175

PA147: 1021 ccggttgaagaagtagaagaagtcgacactgacatctga 1059
            |||||||||||||| |||||||  |||||||| ||||||
P.flu: 1176 ccggttgaagaagtggaagaagctgacactgatatctga 1214
```

Figure A4.1. **a)** Open reading frame of *P. aureofaciens recA*. Predicted start and stop codons are shown in bold. **b)** Best matches using Blastn. **c)** Alignment of *P. aureofaciens recA* (PA147) with *P. fluorescens recA* (P.flu), accession number M96558.1.

finT

```
   1  ATGACATTTC GTCGCCGTTG GGACATCAAC ACCCGGACCC AGCTCATTAC
  51  CCTGGGCCCG GCACTCCTGC TGACGCTGCT GTTGATCAGC TTCTTCACCT
 101  TCGTGCGGAT CCAGGACCTG CGCCAGGAAC TGGACCACAC CGGCCAGTTC
 151  ATCGCCAACC AACTGGCGCC GGCCACCGAA TACGGGGTGA TTTCCGGCAA
 201  CAACGACGTG CTTGAAAGCC TGTTGCGGGC CACCCTCGCC ACGCCGCACG
 251  TGCGCTCCTG GAGATTCAGG ACATGCTGCA GAAAATATCC TGGTGTATGT
 301  CGAGCAACCG TCGGAGAAGC ACGATCGCTC GCTGTCGGTG AAAGTCTTCC
 351  AGGCGCCGAT TCGCTGCAAC ATATCCAGCT GGGCAATGAC TTCTTCCAGG
 401  ACAGTACTGC CGAGCCCAAG GCCCCGCGCG CGGACTACCT GGGGCGAGTG
 451  ATTGTCGGCA TGTCCAACGA TGCGTTCAGC CAGCGCCAGC AGGAAATCCT
 501  GTTCAAGGCC GGGATTCTCG CGCTGTCGCC CTGCTGTTTA CCTTCTGCTG
 551  GCCCGGCGCC TGGCGCCAGC CTGTCGCAAC CGATCAGCGC CATGGGCAAT
 601  GCGGTCAAGG CGATCCAGCA GGGCGATTAC CAGACGCCGC TGCCCATCGT
 651  GGATGACTCG GAGCTGGGCA CCTGTCGCCA CATCAATAAC CTCGCCGATG
 701  CTTTAACCAG GCCAGTCGTG AACAGCACCA GGCCATGGCC AGGTTTATCC
 751  AGACCCGCGA AGAAGCCGAG CGGCAACAAT GCCAAGTCGG ACTTCCTGGC
 801  AATGATGAGC CATGAATTGC GCACGCCGAT GAACGGGGTG CTGGGCATGC
 851  TGCAACTGCT GGAAACCACC GACATGACCG AGGAACAGAC CGAATACGCG
 901  GCGCTGGCCT CGGAAGTCCA CCGAACACCT GCTGAAGGTG ATCAACGACA
 951  TCCTCGACTT CTCGCGCATC GAGCAGGACC CCTGGAGCTT GAGCACATTC
1001  CGTTCGACCT GGTGGAGCTG ATCGGCAGTT GCGCCCAGGC CTTCCAGCAC
1051  GCCGCCCAAC AACGCGGGCT GGCGCTCGAA GTGCCGATCC CCCAGGGCCT
1101  GGGCTCGCTG CAGGTACAGG GCGACCCGAC CCGCATCCGG CAGATTTTGG
1151  TGAACCTGAT CGGCAATGCC CTGAAGTTCA CCGAGCAGGG CACCGTCACC
1201  GTCGAACCCC ACTGGCAGAC CCTGGACCAT GAATTGCTGT GGTTCACCTG
1251  CACCGTACGC GATAGCGGGA TTGGCATTTC CGCCGAGCGC CTGGAACTGA
1301  TGTTCGATGC GTTCCAGCAA GCCGACAGTT CCATTTCAAG ACGTTACGGC
1351  GGCACCGGAC TGGGACTGCC TATCGCACGC ACCCTGGCCG AACGCATGGG
1401  CGGCACCCTG CGCGCCCAGA GCGAAGAAGG CCACGGCTCG GTGTTCACCC
1451  TGGAAATCCC GCTGGCGATC TACCAGCAGA GCTTGCCGGT GCTCGCGCCG
1501  AATACCGAAG GCAATGGCCG TGCGGGTGAA GGGCGCAACG TGTTGCTGGT
1551  GGAAGACAAC CCGGTCAATC GCACGGTGGT CGAAGCATGC TTGCGCAGCC
1601  TGGGGTTCGA GGTGAGCATC GCCACCGACG GCGCCGAAGC GATTCGCAGC
1651  GCCGAGAGCC TGATTTTTAC CGCAATCCTG ATGGACTGCC GACTGCCGGG
1701  CATCGATGGC TACGAGGCTA CCCGGCAGAT TCGCCAGTTG CCCGGTTGCG
1751  CCGAGCTGCC AATCATTGCC TCACGGCCAA TGCTTGCAGG GCGATCGGGA
1801  AGCCTGCCTG GCAGCTGGAA TGAACGATTA CCTGCAAAGC CGTTCAAACG
1851  CACGGATTTG CAGCAAATCC TGCAGAGATG GGTGCAGTAA
```

Figure A4.2. Open reading frame of *finT*. Predicted start and stop codons are shown in bold.

finR and finA

3′ TCCGTAGGGCTCACCTTACCGCGCGAAACGGTGCAGTAGACT**AGT**TTCGTTAAG
CGAATCTTTAAGCTACTTGCGTAACTGAGAAGATCTCGAAGATCTCGGAGCTATTAACCC
GTCTATTTCGCGACTCTACCGGCTCGAGCGCGATAACCCCAACTGGTGTTTTCTAAGTTT
GTTCCTGCAGTCGGAAGGCGAAAGCTATAGAAGCGACTGCTCGGTCAGTCCGTTGTCACG
GTGCGGTGGCGGACGGTCACGCTGAAGCGCGTTCTGAAGCCGTAACAATGAAAACTTGGC
CGGTAGTTGGCTATGCGCCTGGAGGGGTGACAGAAGGACTTTCCAGGTCTGGACGACGAC
TCCCGGTATCGACTTCGCGTATGTCAGTACAACGTTGTCCAGCCAACCGACGAAACCTTT
CGGCGCATGCAGGTCCATAAGCCCCGACCGCTGATGAAAAGCCGCTTTGCATGGATACCG
CGCCGACTGGTGAGGCAGGTACCAACGACGACAAGGGTCAGACTATCTTTCTAGCTATGC
GAGTGGCTCGTTTAGCTGCTACAGTAGGCTGTTCCAGTATAGATTAAGTTCCAGACCTAT
CGCGACATCTTTACGTGGCTATACTCCCGACTAGTTAGCAGCAGGTTTGCGCTGTCCGTG
ACTTTATGCATTAACGGGCCATCCACGTAGTGGGGCTAGGCACTGGCTGCGTAGTCGCAG
TCGGTACCGTGAGTCTTAAAGGGCTCGGAAGTTTATTATACGCCTCGGGCGCAGTCTGTT
CCACTGAACTGCCCAACTCGCTAACTCGTTATGTCGCGGATCAAGCCGAAGGTCGGGTAG
CTAGTAAGCGCAATGGCTTCTCCAACGGTCCGGGTTAACAGACCGGCGTCTACTTTTCGG

AACACTCCT
GAACCTCAGCTGCCAGACGTGCTTGTAGAAGTCCCGGTCGTCGAACAG**GTA***TTGTGAGGA*
finR ◀— 5′

TAAACGGTATCAGCGCGTCTGTTGGTGCAGTTGATAGCGATCAGAGATTCTTCTCTGACC
ATTTGCCATAGTCGCGCAGACAACCACGTCAACTATCGCTAGTCTCTAAGAAGAGACTGG

GCGCATGTCGAGATCCGCAGGGTGCTTTGCGCATTGCGCAAGATTGTGTTGCAGATCGCA
CGCGTACAGCTCTAGGCGTCCCACGAAACGCGTAACGCGTTCTAACACAACGTCTAGCGT

CCGATTCTCCTGTTGAGACAGCGCCGCTACCTTGCACGCCTGAAACCTATTCATGCGCGG
GGCTAAGAGGACAACTCTGTCGCGGCGATGGAACGTGCGGACTTTGGATAAGTACGCGCC
5′—▶ *finA*
*CGTAGCCCGCTTATCTACAGGAAAGCCT***ATG**AAACTTTTCGACCCCATTACAATTGGCAC
GCATCGGGCGAATAGATGTCCTTTCGGA

TCACACTCTCAAGCATCGTATCGCGATGGCTCCCCTGACGCGCTCGCGTGCCGGTCAACC
AGGTAACGTGCCGACGCAGTTGAATGTCGAGTATTACCGTCAACGTGCAAGCGCCGCGCT
AATCATCACAGAGGCTTCTCAAATTTCACAGCAGGGTCAAGGTTACGCCTGGACTCCAGG
TATCTACACGCCCGAGCAAGTTGCTGGATGGAAGGCCGTCAGCGATGCCGTTCATGCCGA
AGGCGGTACGATATTCTTGCAGCTTTGGCACGTGGGTCGTATCTCCCATCCCAGTTTTCA
ACCCAACGGTGGATTGCCGGTCGGGCCGAGTGCTTTGAGTGTAGAGGGCGGCAAGACATT
CATTATTGATGAAGAAGGCAATGGCGTCTGGGGTGACGTTCCAGTACCACAGGCCCTGAC
AATCGAGGGTATCCAGTCAATTATTCAGGACTACCGTGTTGCCGCGCGTAATGCTGTACT
GGCGGGTATGGATGGCGTAGAAATCCATTCTGGCAATGGGTATCTGCTAGACCAGTTCAT
CGATTCCAACAGCAATAAGCGTGATGATATTTACGGAGGGAGTATTGAGAATCGTGCTCG
ATTTTTGCTCGAAGTCACTCAGGCAGTGATTGAGGAAGTGGGGGCTCACCGTGTCGCGGT
ACGCCTGACGCCCATGGGGCGTTTCATGGGCATGGGAGATGCGACACCAGAAGCGACATT
CGGTTATGTGGTGACGCAGCTCAACACGATGAATCTGGCCTATCTGCACCTGGTCGAACC
GGTCATTGTGGGCATCGAGCGAGATGAAAACTTCGATCCGCGCTGGGACACGATCATCAA
GCAGCTTCGTGAGAAGTTACAGCGGTGTGCC**TGA** 3′

Figure A4.3. DNA sequences of *finR* (903bases) and *finA* (906 bases) shown as arranged in PA147-2. Bold type indicates putative start and stop codons. Both strands of the intergenic region (italics) are shown. Only the coding strands of *finR* and *finA* sequences are shown, and these are indicated with 5' and 3' annotation, and arrows to show coding direction.

A4.2 Sequences flanking transposon insertion points

pCM1

GCTGGGTCCGGGTGTTGATGTCCCAACGGCGACGAAATGTCATGGGCGTTTTTCTCCTTCGGCCAGGCGGGCG
GCGACGTTGGTTTCGTCCACCTGTTCGATCCCCAGTGAGCGCGCAACCTGCGGGTTGCCTACGACTTTGAAAC
GTTCCGGGTACAGCGAGCGCGGCCAACTGGCCGGCGCATGATCCAGCAGGCGGTCGAGCACATCCAGCCAGTC
GGTCTGATCGCTGTAGGTGCTGGCCAGGCTGCCGGCCTTGACGAACCCGGCGTTGGGCCCGACCAGTGGCAAT
TGCCTGGCGTAGCTGCTGAGCAGCAGGTTTTTCGCGGTTTTCGGGTTGTACAGCTGGGGATCATCCAGCCCCA
GCAAGACGTCGCTGTTCTTGAACAGGCTTTGCAGCGGGCGACTGTCATTAATGTTTTCCCAAAGCTGGGGCAC
GATCTCCAAGCCCATGGGCGCGGCGTACTGGCGCAGATCGCGCAGNANGAACTCGCTGTCGATGCCATAAANC
ACGCCGATTNCGCCCGGGCCTTGAGGCNNGAATGCCCGGTNAATCAAGGCNCANTTTGTTCGGTTCCCANNCN
GNTTGGGGTTCCNTCCCACAANNCAAGNGCTTGGATTCTTTCCGGCAANGGCAAA

pCMH26

AGCCAGCGCTTTTCAGGCGCTCGNGGGGCACTTCTTCTTTCTGCACCACTTCGCCGACGACGATGTGATTTTC
CTGGCCCGGCACGTTGTAGTTGGCCTGGATCAGGTCGGCCGGCCAGAAGTGGCCCAGGCCACGCACGGCAATC
ACGGCAAGCAAACCAATGGTCATGATGACCGCGATGGACACCGCGCCACCGCTGATCCAGACGCCCGGGGCGC
CGCTCTTGAACCATCCATTCAGGGAGTTCTGTTTCACAGACTTCTACCTTTCTTAAAGCGACGAGTATTTCTT
GCGCAGACGCTGACGAATCAGCTCGGCGAGGGTGTTCATGATGAAGGTGAACAGCAGCAGCACCAGCGCCGAG
AGGAACAGCACGCGGTAGTGGCTGCCGCCGACTTCCGATTCGGGCATTTCCACCGCGACGTTGGCTGCCAGGG
TGCGCAGGCCTTCAAACAGGTTCATCTCCATCACCGGCGTGTTGCCGGTGGCCATCAGCACGATCATGGTTTA
CCGACCGNCGGCCCATGCCCGATCATCAGCGCCGAGAAGATGCCTGGGCTTGGNCGGGTCAAGGATCACCGAC
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pCMI75

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pCMI95

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pCM168

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A4.3 Blastx results for searches with *finR* and *finT* (highest 15 matches)

finR

Sequences producing significant alignments:	(bits)	Value
gi|11352205|pir||B83328 probable transcriptional regulator ...	185	8e-46
gi|11352243|pir||B83256 probable transcriptional regulator ...	182	7e-45
gi|3169721|gb|AAC17939.1| (AF007569) GstR [Bradyrhizobium j...	181	2e-44
gi|13473471|ref|NP_105038.1| transcriptional regulator [Mes...	181	2e-44
gi|7448775|pir||S77111 transcription regulator slr1871 - Sy...	179	6e-44
gi|401473|sp|P30864|YAFC_ECOLI HYPOTHETICAL TRANSCRIPTIONAL...	176	3e-43
gi|13473045|ref|NP_104612.1| transcriptional regulator, Gst...	174	1e-42
gi|12512936|gb|AAG54504.1|AE005196_2 (AE005196) putative tr...	174	1e-42
gi|7448774|pir||S74869 transcription regulator slr1245 - Sy...	171	1e-41
gi|11352056|pir||F83412 probable transcription regulator PA...	170	3e-41
gi|11279620|pir||A82391 transcription regulator LysR family...	169	6e-41
gi|13472475|ref|NP_104042.1| transcriptional regulator [Mes...	168	8e-41
gi|13471829|ref|NP_103396.1| probable transcriptional regul...	167	2e-40
gi|13476459|ref|NP_108029.1| transcriptional regulator [Mes...	166	3e-40
gi|11351944|pir||F83638 probable transcription regulator PA...	166	5e-40

finT

Sequences producing significant alignments:	(bits)	Value
gi|11351825|pir||E83443 probable sensor/response regulator ...	593	e-168
gi|13421116|gb|AAK22014.1| (AE005677) sensory box histidine...	221	2e-56
gi|13424758|gb|AAK25064.1| (AE005973) sensor histidine kina...	214	4e-54
gi|7444070|pir||S75136 sensory transduction histidine kinas...	212	1e-53
gi|13424253|gb|AAK24637.1| (AE005934) sensory box histidine...	209	9e-53
gi|13424056|gb|AAK24472.1| (AE005918) sensory box histidine...	208	2e-52
gi|11356137|pir||C82151 sensor histidine kinase VC1831 [imp...	206	8e-52
gi|13424622|gb|AAK24950.1| (AE005962) sensor histidine kina...	204	2e-51
gi|11352379|pir||D83149 probable two-component sensor PA397...	200	4e-50
gi|13424601|gb|AAK24933.1| (AE005960) sensory box histidine...	200	6e-50
gi|11356143|pir||E82198 sensor histidine kinase/response re...	199	1e-49
gi|13424725|gb|AAK25037.1| (AE005970) sensor histidine kina...	198	2e-49
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gi|11351830|pir||H83132 probable sensor/response regulator ...	197	5e-49
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Appendix 5

Published papers

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Biological control of *Phytophthora megasperma* var. *sojae*, causal agent of Phytophthora rot of asparagus, by *Pseudomonas aureofaciens* PA147-2: a preliminary field trial

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Abstract *Pseudomonas aureofaciens* PA147-2 produces an antibiotic that inhibits the growth of *Phytophthora megasperma* var. *sojae*, the causative agent of root-rot disease of asparagus (*Asparagus officinalis*). To assess the potential use of PA147-2 as a biocontrol organism, we report preliminary findings of a field application of PA147-2 to asparagus crowns. To determine the ability of PA147-2 to suppress Phytophthora rot, asparagus crowns inoculated with PA147-2 were planted in Eyre shallow fine sandy loam soil inoculated with *P. megasperma* var. *sojae*. During the 6-month trials, PA147-2 was isolated from inoculation points with a decreasing frequency. Harvesting and analysis of asparagus plants revealed that inoculation of crowns with PA147-2 resulted in a 55.8% increase of fern dry weight in comparison to untreated plants. Preliminary field trial results suggest the direct inoculation of asparagus crowns with PA147-2 before planting causes a statistically significant increase in the yield of plant material in the presence of *P. megasperma* var. *sojae*.

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Keywords *Pseudomonas aureofaciens*; *Phytophthora megasperma* var. *sojae*; *Asparagus officinalis*; biocontrol; bacterial survival; Ridomil[90] 250EC

INTRODUCTION

Phytophthora root rot causes significant yield reduction of asparagus (*Asparagus officinalis*) crops throughout New Zealand (Falloon 1991). *Phytophthora megasperma* var. *sojae* has been isolated from asparagus fields throughout the main growing areas within New Zealand (Boesewinkle 1974; Falloon 1982). Asparagus growers currently depend on chemical fungicides for control of the disease. However, many chemical pesticides are not specific for a particular organism, and can therefore have harmful effects on beneficial micro-organisms in the soil (Heger et al. 1995). Research directed toward non-chemical alternatives which continue to maintain high crop yields is desirable (Cook et al. 1995).

Fluorescent pseudomonads are suitable biocontrol antagonists in part because of their adaptive metabolism and ability to produce a wide range of secondary metabolites inhibitory toward plant pathogens (Leisinger & Margraff 1979; Thomashow & Weller 1988). Pseudomonads are common inhabitants of the rhizosphere where they contribute to the control of many deleterious fungal pathogens (Fravel 1988; Lievens et al. 1989).

In a previous study, a bacterium from soil with *in vitro* antifungal activity was identified as a strain of *Pseudomonas aureofaciens* and was designated PA147-2 (Carruthers et al. 1994). Glasshouse trials indicated that suppression of root rot of *A. officinalis* seedlings caused by *P. megasperma* var. *sojae* was possible by PA147-2 (Carruthers et al. 1995). Here we describe a field trial in which the ability of PA147-2 to suppress *P. megasperma* var. *sojae* root rot of asparagus plants was assessed. The trial was designed to address whether biocontrol could provide comparable protection achieved by current

chemical control strategies (Baker 1987). To address this question, four experimental treatments were used to enable a direct analysis of the degree of protection PA147-2 provided against Phytophthora root rot of asparagus under field conditions. Results of our trial are presented in this paper.

MATERIALS AND METHODS

Trial design

The trial was carried out over 6 months (September 1996–March 1997) in an isolated area of Eyre shallow fine sandy loam soil inoculated with *P. megasperma* var. *sojae* using 22-month-old asparagus crowns (cultivar 'UC157' F1 hybrid) previously grown in Templeton silt loam soil. Planting of treated crowns was performed using a randomised complete block design (Steel & Torrie 1976). Each plot consisted of 20 crowns planted 300 mm apart within a single row at a depth of 80 mm. Each treatment was replicated 4 times. Dams (200 mm high) were erected at the end of each plot to prevent leaching and cross-contamination of treatments. Guard rows were planted on the sides of the trial. Randomly selected crowns in the guard rows were inoculated with PA147-2 for use in the population study experiment. General maintenance of plants consisted of 25 mm of irrigation every 14 days applied over a 3-h period. Because saturated or near saturated soil conditions are essential for *Phytophthora* zoospore production and dispersal (Falloon & Tate 1986), flooding of the plot was achieved by irrigating for extended time periods (Table 1). Fertiliser, insecticides, and herbicides were applied as indicated in Table 2.

Harvest was carried out 160 days after planting. The fern from each plot was cut at soil level, washed with tap water to remove soil particles and placed into paper bags. Following oven drying at 75°C for 72 h, dry weights of the fern were determined.

Experimental treatments

Four experimental treatments were used. Treatment 1 was the application of a commercially available fungicide, Ridomil® 250EC, which is applied for the control of Phytophthora rot of asparagus throughout New Zealand. In Treatment 2, asparagus crowns were left untreated. Treatment 3 was the inoculation of asparagus crowns with rifampicin resistant (Rif^R) PA147-2. The fourth treatment was the application of Luria Bertani (LB) medium (Sambrook et al. 1989) to crowns. The four treatments allowed the comparison of PA147-2 treatment to a current chemical control, and to asparagus that has no "added protection". In addition, any unexpected effect of the bacterial growth medium could be assessed.

Treatment of crowns

Rif^R PA147-2 was cultured at 30°C in LB media to a viable count of 1×10^8 CFU ml^{-1} (250 rpm 24 h). The culture was maintained on ice during transportation to the field. Asparagus crowns were inoculated by submergence in a suspension of PA147-2 for 25

Table 1 Watering schedule applied to asparagus (*Asparagus officinalis*) field trial plot.

Date (1996)	Irrigation (mm)	Time period (h)
26 Sep	100	8
27 Sep	200	16
17 Oct	200	16
18 Oct	200	16
24 Oct	100	8
25 Oct	200	16

Table 2 Chemicals applied to asparagus (*Asparagus officinalis*) field trial plot.

Date	Chemical applied	Description	Amount applied (kg ha^{-1})
2 Oct 1996	Karmex	Herbicide	2
11 Dec 1996	Malathion	Insecticide	2
13 Dec 1996	Linuron	Herbicide	1
14 Dec 1996	Crop Master 13	Fertiliser	350
18 Jan 1997	Malathion	Insecticide	2
2 Feb 1997	Malathion	Insecticide	2
16 Feb 1997	Malathion	Insecticide	2

min with gentle agitation. Untreated controls were dipped in sterile distilled water (dH_2O), and LB treatment was carried out by dipping crowns in the medium. Crowns were planted within 30 min of treatment.

Bacterial population study

Aerobic, Rif^R bacteria were isolated at 2-week intervals from the rhizosphere and soil surrounding the rhizosphere (non-rhizosphere) from both PA147-2 inoculated and control crowns within guard rows. Random soil samples of 1 g each were collected from each sample area and placed in 100 ml of sterile dH_2O containing 10 g of sterile gravel (7 mm coarse chip). Following agitation (250 rpm for 20 min), serial 10-fold dilutions were spread-plated on LB agar supplemented with 50 μg ml^{-1} rifampicin (Rif) and 100 μg ml^{-1} cycloheximide (Cx) and incubated (30°C for 24 h). Culturable rifampicin resistant (Rif^R) colony forming units per gram (cfu g^{-1}) were determined for rhizosphere and non-rhizosphere samples and the mean determined for the four samples.

Rif^R isolate analysis

The following procedures used a –80°C stock of the original PA147-2 inoculation culture as a comparative control in all experiments. Colony morphology of Rif^R isolates was compared using a stereo microscope and cellular morphology was observed by Gram staining. Bioassays against *P. megasperma* var. *sojae* were carried out on phosphate buffered potato dextrose agar (PBPDA) as outlined by Carruthers et al. (1994). Fluorescent siderophore production was determined by incubation (30°C for 72 h) of isolates on Pseudomonas Minimal Media (PMM) (Kirner et al. 1996) followed by viewing under UV illumination (260 nm). Antibiotic resistance of isolates was determined by replica-plating onto LB agar supplemented with either rifampicin (Rif, 50 μg/ml), ampicillin (Ap, 100 μg/ml), kanamycin (Km, 50 μg/ml), gentamicin (Gm, 15 μg/ml), naladixic acid (Nal, 30 μg/ml), streptomycin (Strep, 50 μg/ml), or tetracycline (Tc, 15 μg/ml) and following incubation for 24–72 h at 30°C, bacterial growth was assessed. Analytical Profile Index (API) 20 NE strip analysis was performed according to the manufacturer's protocol.

Southern hybridisation

Southern hybridisation was performed on PA147-2 isolated from treated crowns to indicate the previously identified antifungal region (Carruthers et al. 1994) was present, thus supporting the identification as PA147-2. Extraction and preliminary purification of plasmid DNA (pFC900B) for use as a probe was carried out using the alkaline lysis technique (Birnboim & Dolly 1979) and the GENECLEAN® III kit (BIO 101, Inc.) was used according to manufacturer's recommendations for final purification before labeling. Genomic DNA from isolates, and PA147-2 were prepared using a method derived from Scott et al. (1981). Restriction endonuclease digestion using *Eco*RI (Boehringer) of the respective genomic DNA was carried out according to enzyme manufacturer's recommendations. Separation of digested fragments was performed using agarose gel electrophoresis (1% agarose) and Southern transfer of DNA fragments to Hybond N+ membrane (Amersham) was carried out using the Pharmacia LKB VacuGene XL vacuum blotting system (Pharmacia). Probe labeling of pFC900B, hybridisation, and signal detection of membranes was done using the non-radioactive enhanced chemiluminescence (ECL™) kit (Amersham).

Harvest statistical analysis

After harvest, individual plants were visually graded on percentage of diseased foliage using a scale of 1–5 (1 = 0%, 2 = 1–20%, 3 = 21–40%, 4 = 41–80%, 5 = 81–100%) and analysed by fitting an ordinal logistic model. Fern dry weights for each treatment were analysed for significant differences using a one-sided test.

RESULTS

Disease severity

Phytophthora megasperma var. *sojae* disease symptoms were observed on asparagus plants at harvest (Fig. 2), indicating that *Phytophthora* was well established. Symptoms ranged from reduced fern vigour with slight browning of fern tips, to stunted growth and wilting with browning over most of the plant. When visual disease severity data was analysed using an ordinal logistic model it was found that there was no significant heterogeneity, so treatment effects were tested against theoretical multinomial values. Fitted model coefficient for Ridomil® 250EC, PA147-2, and LB were –1.1, –0.9, and –0.2 respectively. The standard error of these coefficients was 0.30. Fern dry weights are shown in Table 3. Visual observation of the trial plants indicated that treatment of asparagus crowns with Ridomil® 250EC provided a high degree of protection against

Phytophthora rot. In contrast, the untreated and LB treated controls were severely affected by Phytophthora rot, with all plants showing some degree of disease. The plants treated with PA147-2 showed signs of disease, although the symptoms were significantly less severe than those displayed by the untreated plants.

Effect of PA147-2 on disease severity

PA147-2 improved dry weights of fern by almost 60% (significant at 5% level using a one-sided test), but Ridomil® 250EC improved dry weights by much more, averaging 173% more than untreated plants.

Bacterial population study

Untreated crowns

Comparison of Rif^R isolates from untreated crowns revealed four morphologically-dissimilar colony groups which API 20 NE strip analysis revealed to be three *P. fluorescens* strains (0147475, 0147755, and 0147775) and one *P. aureofaciens* strain (0147577). Bioassay revealed one of the three *P. fluorescens* strains (API 20 NE profile 0147755) to have antifungal activity toward *P. megasperma* var. *sojae*.

PA147-2 treated crowns

Culturable Rif^R CFU g^{-1} of bacteria isolated from PA147-2 inoculated crowns are presented as a logarithmic linear graph with comparison to Rif^R CFU g^{-1} isolated from control crowns (Fig. 1).

Comparison of all Rif^R bacterial isolates from PA147-2 inoculated crowns indicated six morphologically-distinguishable colony types of which a single representative of each was purified onto PMM and labelled as PA147-2 crown isolate (PACI) A–F. When PACI isolates A–F were bioassayed against *P. megasperma* var. *sojae*, all demonstrated antifungal activity comparable to PA147-2 after 7 days.

Table 3 Summary of asparagus (*Asparagus officinalis*) fern dry weights used in statistical analysis. (LB = Luria Bertani medium; PA147-2 = *Pseudomonas aureofaciens* PA147-2.)

Treatment	Weight (g)
Untreated	160
Ridomil® 250EC	437
PA147-2	252
LB	186
LSD (5%), 7 d.f.	109

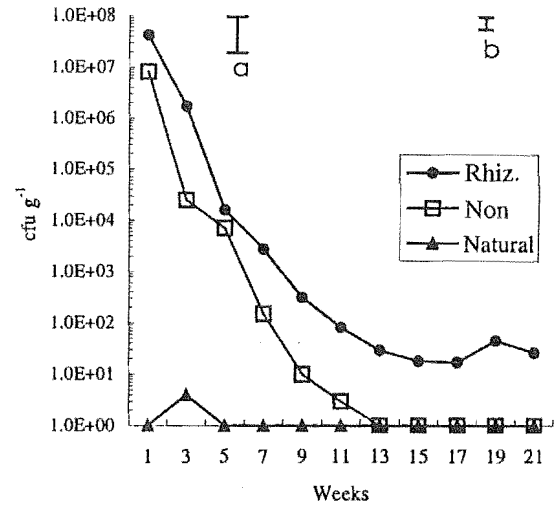

Fig. 1 Rifampicin-resistant bacteria isolated during the field trial. (Rhiz. = bacteria isolated from the rhizosphere of inoculated plants; non = bacteria isolated from soil surrounding the roots of inoculated plants; natural line shows the rifampicin-resistant bacteria isolated from control plants that were not inoculated with PA147-2.) Data presented are the mean from four samples (a = LSD (5%) for comparing rhiz. with non-rhiz. positions at any of Weeks 1–11 (d.f. =18); b = LSD (5%) for comparing rhiz. mean with zero, for Weeks 13–21 (d.f. = 15).

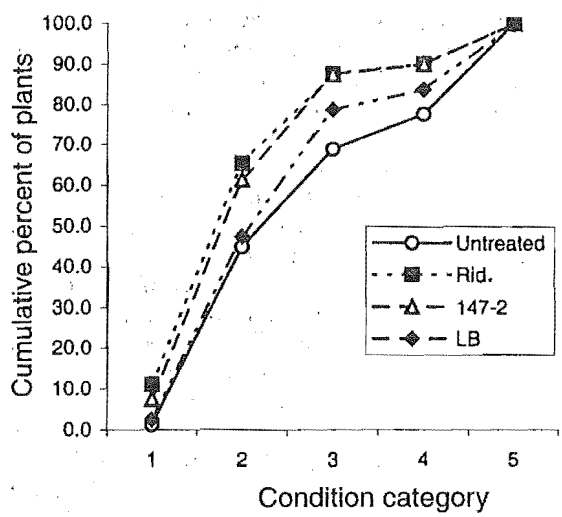

Fig. 2 Disease severity visually observed from asparagus (*Asparagus officinalis*) plants growing in diseased plot. Individual plants were visually graded on overall plant condition on a scale of 1–5 to indicate percentage of foliage effected by disease (1 = 0%, 2 = 1–20%, 3 = 21–40%, 4 = 41–80%, and 5 = 81–100%). (Rid. = Ridomil®; LB = Luria Bertani medium; 147-2 = *Pseudomonas aureofaciens* PA147-2.)

PACI isolates A–F exhibited siderophore production on PMM agar after 5 days with equal fluorescence intensities to PA147-2. LB agar supplemented with the respective antibiotic revealed that all PACI isolates A–F had resistance to Rif and Ap but were sensitive to Km, Gm, Nal, Strep, and Tc, like PA147-2. Gram stain preparation of PACI isolates A–F revealed Gram-negative rods, identical in size and cellular morphology to PA147-2. Biotypes obtained using API 20 NE were identical for PACI isolates A–F and PA147-2 (0147577). 0147577 is 83% confirmation of a "very good identification to the genus *P. aureofaciens*" (1996 API Software Database). Southern hybridisation analysis using the ECL™ kit (Amersham) indicated hybridisation of the 16kb antifungal region contained in pFC900B to PACI isolates A–F and PA147-2 within an identically-sized *Eco*RI DNA fragment, supporting the identification of the PACI isolates as PA147-2 (data not shown).

DISCUSSION

The field trial presented in this study was carried out to see whether inhibition of Phytophthora rot of asparagus by PA147-2 observed in glasshouse trials (Carruthers et al. 1995) could also be demonstrated under field conditions, and focused on preliminary findings of the field application of PA147-2 as a biocontrol agent against *P. megasperma* var. *sojae*.

Fern dry weight was used as a measure of plant vigor because fern vigor of asparagus plants in the first 3 years of planting is proportional to the yield of spears obtainable over future years (Scheer & Ellison 1960). The percentage increase in total fern dry weight obtained from plants treated with Ridomil® 250EC and PA147-2 was calculated in comparison to the fern dry weight obtained from untreated plants. Dry weight increases relative to the untreated control were considered to have arisen from disease suppression provided by the respective treatment. Treatment with Ridomil® 250EC and PA147-2 resulted in dry weight increases of 173 and 55.8% respectively. Although Ridomil® 250EC shows considerably greater protection than PA147-2, the bacterial treatment is still significantly better than the untreated control (5% LSD). It is interesting to note that treatment with LB resulted in a slightly greater fern weight than the untreated plants. We speculate that the LB may have stimulated growth of indigenous bacteria on the crowns, resulting in either greater competitive exclusion of the pathogen, or production of antifungal metabolites by the resident microbes. This effect would be short lived because the LB concentration would reduce quickly after planting and watering.

One of the plots treated with Ridomil® 250EC only provided 21% of its expected dry weight of fern, after adjusting for systematic local variation (which comprised block effects and a 30% reduction of fern dry weight in plots along one edge of the trial near some shelter trees), so data from this plot were excluded from statistical analysis of fern dry weights.

During the field trial, monitoring of PA147-2 survival was achieved by periodic re-isolation. Based on differences in colony morphology, initial doubt was raised as to whether all Rif^R bacteria isolated from crowns inoculated with PA147-2 were the same *P. aureofaciens* PA147-2 strain used for inoculation. Subsequent phenotypic analysis indicated that those Rif^R isolates chosen for analysis were identical to PA147-2 and Southern hybridisation indicated genetic similarity to a 16kb genomic region within PA147-2 believed to be responsible for antifungal activity (Carruthers et al. 1994). The result of the Southern hybridisation supports the identification of the PACI isolates as PA147-2, and that the PACI isolates still inhibit the *in vitro* growth *of P. megasperma* suggests that the bacteria were probably active in biocontrol in the rhizosphere. Although not all Rif^R bacteria isolated from crowns inoculated with PA147-2 were subjected to Southern hybridisation, the number of Rif^R bacteria isolated from crowns inoculated with PA147-2 far exceeded natural background levels of Rif^R bacteria therefore suggesting Rif^R bacteria isolated were an accurate estimation of PA147-2 survival (Fig. 1).

Results of this field study have indicated that the direct inoculation of asparagus crowns with PA147-2 before planting resulted in a 55.8% increase in fern yield compared to the untreated control. Because disease control is essential during wet field conditions when *Phytophthora* zoospore production and dispersal is maximal (Falloon et al. 1985), the results of the bacterial population study suggest that PA147-2 survival was sufficient during the trial to enable fungal inhibition by either: (1) the production of a sufficient quantity of antifungal compounds resulting in inhibition of *P. megasperma* var. *sojae* establishment; or (2) the inhibition of the pathogen by the introduction of microbial competition because of the establishment of PA147-2 in the rhizosphere. Specific experiments could be designed to examine which of the above mechanisms is responsible for disease suppression, or whether suppression results

from the combined action of antibiotic production and microbial competition.

The observation that asparagus plants inoculated with PA147-2 showed a 55.8% increase in fern dry weight compared to untreated plants indicated the potential of PA147-2 as a biocontrol agent. However the fungicide Ridomil® 250EC, provided a 173% increase and therefore re-emphasises that biological control of Phytophthora rot of asparagus with *P. aureofaciens* still needs extensive investigation before becoming a viable option that provides comparable protection to that given presently by chemical pesticides. As a result of the increasing demand being generated in the area of organic farming (Figiel 1994), PA147-2 application may provide an alternative non-chemical option resulting in an increase in crop yields. Application of PA147-2 at the time of planting will provide protection to the plants as they become established, but the bacterial population study suggests that the bacterial numbers will be very low by the time of harvest which is important when considering the issue of human consumption. In addition, the use of an integrated strategy of pest management in which PA147-2 is used in combination with a chemical fungicide may allow a reduction in the amount of chemicals applied to crops. Although such an approach would not eliminate the use of chemicals, the reduced need for them could have both economic and environmental benefits. Future trials would be useful for three reasons. First, further investigations involving larger trials will enable a more extensive analysis of the ability of PA147-2 to inhibit Phytophthora rot of asparagus. Second, it would be useful to isolate the biocontrol agent from experimental plants (rather than guard row plants as in this study), to assess the effects of any interactions between the bacterium and the pathogen. Finally, long-term studies would allow an assessment of whether biological control of disease in the early life of asparagus leads to an increased crop yield when the plants begin to produce harvestable spears.

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REFERENCES

Baker, K. F. 1987: Evolving concepts of biological control of plant pathogens. *Annual Review of Phytopathology 25*: 67–85.

Birnboim, H. C.; Dolly, J. 1979: A rapid alkaline extraction procedure for screening recombinant plasmid DNA. *Nucleic Acids Research 7*: 1513–1523.

Boesewinkle, H. J. 1974: *Phytophthora* on asparagus in New Zealand. *New Zealand Plant Disease Reporter 58*: 525–529.

Carruthers, F. L.; Conner, A. J.; Mahanty, H. K. 1994: Identification of a genetic locus in *Pseudomonas aureofaciens* involved in fungal inhibition. *Applied and Environmental Microbiology 60*: 71–77.

Carruthers, F. L.; Shum-Thomas, T.; Conner, A. J.; Mahanty, H. K. 1995: The significance of antibiotic production by *Pseudomonas aureofaciens* PA147-2 for biological control of *Phytophthora megasperma* root rot of asparagus. *Plant and Soil 170*: 339–344.

Cook, R. J.; Gabriel, C. J.; Kelman, A. 1995: Research on plant disease and pest management is essential to sustainable agriculture. *BioScience 45*: 354–357.

Ellison, J. H. 1986: Asparagus breeding. *In*: Breeding vegetable crops. New York, AVI Publishing Co. Pp. 521–569.

Falloon, P. G. 1982: Baiting, pathogenicity, and distribution of *Phytophthora megasperma* var. *sojae* in New Zealand asparagus soils. *New Zealand Journal of Agricultural Research 25*: 425–429.

Falloon, P. G. 1991: Effect of root temperature, plant age, frequency and duration of flooding and inoculum placement and concentration on susceptibility of asparagus to Phytophthora rot. *New Zealand Journal of Crop and Horticultural Science 19*: 305–312.

Falloon, P. G.; Mullen, R. J.; Benson, B. L.; Grogan, R. G. 1985: Control of Phytophthora rot with metalaxyl in established asparagus. *Plant Disease 69*: 921–923.

Falloon, P. G.; Tate, K. G. 1986: Major diseases of asparagus in New Zealand. *Proceedings of the Agronomy Society of New Zealand 16*: 17–28.

Figiel, R. 1994: Vintners take the pledge—grape growers and wine makers switch from using chemical pesticides to organic farming methods. *Sierra 79*: 51–55.

Fravel, D. R. 1988: Role of antibiosis in the biological control of plant diseases. *Annual Review of Phytopathology 26*: 75–91.

Heger, W.; Jung, S.-J.; Martin, S.; Peter, H. 1995: Acute and prolonged toxicity to aquatic organisms of new and existing chemicals and pesticides. *Chemosphere 31*: 2707–2726.

Kirner, S.; Krauss, S.; Sury, G.; Lam, S. T.; Ligon, J. M.; van Pee, K.-H. 1996: The non-haem chloroperoxidase from *Pseudomonas fluorescens* and its relationship to pyrrolnitrin biosynthesis. *Microbiology 142*: 2129–2135.

Leisinger, T.; Margraff, R. 1979: Secondary metabolites of the fluorescent Pseudomonads. *Microbiological Reviews 43*: 422–442.

Lievens, K. H.; Van Rijsbergen, R.; Leyns, F. R.; Lambert, B. J.; Tenning, P.; Swings, J.; Joos, H. J.-P. 1989: Dominant rhizosphere bacteria as a source for antifungal agents. *Pesticide Science 27*: 141–154.

Sambrook, J.; Fritsch, E. F.; Maniatis, T. 1989: Molecular cloning: a laboratory manual. 2nd edition. United States, Cold Spring Harbor Laboratory Press.

Scheer, D. F.; Ellison, J. H. 1960: Asparagus performance as related to seedling vigor. *American Society for Horticultural Science 76*: 370–375.

Scott, J. F. K.; Rolfe, B. G.; Shine, J. 1981: Biological nitrogen fixation: primary structure of the *Klebsiella pneumoniae nif*H and *nif*D Genes. *Journal of Molecular and Applied Genetics 1*: 71–81.

Steel, R. G. D.; Torrie, J. H. 1976: Introduction to statistics. New York, McGraw-Hill.

Thomashow, L. S.; Weller, D. M. 1988: Role of a phenazine antibiotic from *Pseudomonas fluorescens* in biological control of *Gaeumannomyces graminis* var. *tritici*. *Journal of Bacteriology 170*: 3499–3508.

The importance of *recA* mutant strains for the study of antifungal genes in *Pseudomonas aureofaciens* PA147-2

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Abstract: *Pseudomonas aureofaciens* PA147-2 shows antifungal activity toward a variety of plant pathogenic fungi. We have been investigating the molecular mechanisms underlying the fungal inhibition, and during these studies it was observed that the use of pLAFR3-based cosmids for in trans complementation of mutants lacking antifungal activity is hindered by cosmid instability. It was hypothesised that the cosmid stability could be improved by inactivation of *recA*. The *recA* gene of PA147-2 was cloned and shown to complement *recA* mutants of *E. coli*, restoring RecA-dependent functions when expressed in trans. Two *recA* mutants of PA147-2 were constructed. Both of these mutants show sensitivity to DNA damage. Cosmid pPS2122 restores antifungal activity to a mutant by allele exchange, but is unstable in trans. The stability of pPS2122 is shown to be improved in a *recA* mutant of PA147-2 with respect to the wild type.

Key words: antifungal, complementation, *recA*, *Pseudomonas aureofaciens* PA147-2.

Résumé : Le *Pseudomonas aureofaciens* PA147-2 possède une activité antifongique contre une variété de champignons phytopathogènes. Nous nous sommes intéressés aux mécanismes moléculaires de cette inhibition fongique et avons constaté que l'instabilité des cosmides entravait l'utilisation des cosmides basés sur pLAFR-3 pour la complémentation in trans de mutants dépourvus d'activité antifongique. Nous avons émis l'hypothèse que la stabilité des cosmides pouvait être augmentée par l'inactivation de *recA*. Le gène *recA* du PA147-2 a été cloné et il s'est avéré capable de complémenter des mutants *recA* d'*Escherichia coli*, rétablissant les fonctions dépendantes de RecA lorsqu'il est exprimé in trans. Deux mutants *recA* du PA147-2 ont été construits et les deux étaient sensibles à voir leur ADN endommagé. Le cosmide pPS2122 a pu rétablir l'activité antifongique chez un mutant par échange d'allèles mais il était instable in trans. Dans un mutant *recA* du PA147-2, la stabilité du pPS2122 était meilleure que chez le type sauvage.

Mots clés : antifongique, complémentation, *recA*, *Pseudomonas aureofaciens* PA147-2.

[Traduit par la Rédaction]

Introduction

Pseudomonas aureofaciens PA147-2 is a soil bacterium that has potential as a biological control agent against fungal diseases in agriculture. PA147-2 inhibits growth of the phytopathogenic fungi *Phytophthora megasperma* var. *sojae* and *Rhizoctonia solani* in in vitro studies, and has also been shown to suppress *Phytophthora* rot of asparagus in glasshouse trials (Carruthers et al. 1995). We are investigating the molecular basis for the antifungal phenotype of PA147-2; however in our laboratory the frequent loss of pLAFR3-based cosmids from PA147-2 and its mutant derivatives has been observed, hindering the *trans* complementation of mutants (Carruthers et al. 1994). Our interest in the *recA* system of *P. aureofaciens* PA147-2 is two-fold. Firstly, since the loss of cosmids had often been accompanied by allele exchange between the cosmid and the genome, we were interested to examine any possible role of RecA in this phenotype. Secondly, construction of *recA* mutants might facilitate further genetic analysis of PA147-2 and its derivatives by stable in trans complementation experiments.

The recombination-deficient phenotype involving *recA* in *Escherichia coli* was first discovered during Hfr mating experiments, when it was noted that a recipient strain inherited nutritional markers at a lower-than-expected frequency (Clark and Margulies 1965). The defect was shown to be in homologous recombination between the incoming DNA and the recipient genome. RecA is now known to be a multifunctional protein in *E. coli*, with well-characterised roles in homologous recombination (Kowalczykowski et al. 1994), recombinational DNA repair, and induction of the SOS regulon (Miller and Kokjohn 1990). In the first two of these processes, RecA promotes homologous pairing and strand exchange, while in the SOS response RecA acts as a coprotease in the cleavage of the LexA repressor of the SOS regulon. RecA also appears to function in homologous recombination and DNA repair in *Pseudomonas fluorescens* (De Mot et al. 1993), *P. putida* (Luo et al. 1993), and

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Table 1. Bacterial strains, plasmids, and phages used in this study.

Bacterial strains, plasmids, and phages	Genotype or description	Reference or source
E. coli		
DE880	Δ(*lac-argF*)*U169 relA1 thi-1 cps-3 sulA*::Mu d (*lac* Ap)XCam (Mu+) *srlC300*::Tn*10*	(Ennis et al. 1989)
DE1491	Δ(*lac-argF*)*U169 relA1 thi-1 cps-3 malF55*::Tn*5 sulA*::Mu d (*lac* Ap)XCam (Mu+) Δ(*recA-srlR*)301::Tn*10*	(Ennis et al. 1989)
DH5α	*supE44* Δ*lacU169*(ϕ*lacZ*ΔM15) *hsdR17 thi-1 relA1 recA1 endA1 gyrA96*	(Hanahan 1983)
MC4100	*araD139* Δ(*lacIPOZYA-grgF*)*U169 rpsL thi recA56 supO*	(Gilson et al. 1987)
S17-1	*recA294*::Tn*7 tra*$^+$ (contains chromosomally integrated RP4 derivative)	(Simon et al. 1983)
W3110	Wild-type K12	(Kohara et al. 1987)
MT2	*ilv his rpsL recA1* (N7N53cI+)	(Toman et al. 1985)
P. aureofaciens		
PA147-2	Rf^R inhibitor of fungal growth	(Carruthers et al. 1994)
PA147-2recA1	*recA*::miniTn*10* derivative of PA147-2	This study
PA147-2recA4	*recA*::miniTn*10* derivative of PA147-2	This study
Plasmids		
pBLUESCRIPT KS-		Stratagene
pREC1	Cosmid containing *recA* gene of PA147-2	This study
pME6001	Gm^R cloning vector	Stephen Heeb
pREC6g	6kb *Eco*RI/*Hin*dIII *recA*$^+$ clone in pME6001	This study
pREC6M3	MiniTn*10 recA*$^-$ mutant of pREC6g	This study
pPRAM1	MiniTn*10 recA*$^-$ mutant of pREC1	This study
pPRAM4	MiniTn*10 recA*$^-$ mutant of pREC1	This study
pPS2122	Cosmid from PA147-2 library. Complements antifungal minus mutant PA138 by allele exchange	Lab collection
Phages		
λNK1316	Lambda delivery vehicle for miniTn*10* derivative 103	(Kleckner et al. 1991)
$P1_{vir}$		(Silhavy et al. 1984)

P. aeruginosa (Sano and Kageyama 1987). In addition to the functions above, RecA has been shown to be involved in the secretion of an extracellular nuclease by *Serratia marcescens* (Ball et al. 1990), and in the regulation of colicin E1 expression in *E. coli* (Salles et al. 1987). Construction of *recA* mutant strains of *P. syringae* (Willis et al. 1988) and *P. putida* (Luo et al. 1993) resulted in strains that were useful in trans complementation studies.

Here we present a preliminary investigation into the *recA* homolog of PA147-2. We show (*i*) *recA*-dependent functions in *E. coli* can be complemented by a *recA*$^+$ clone from PA147-2, (*ii*) the construction of *recA* mutants of PA147-2, and (*iii*) the demonstration of increased cosmid stability in *recA* deficient *P. aureofaciens* PA147-2.

Bacterial strains, plasmids, and bacteriophages

Bacterial strains, plasmids, and bacteriophages used in this study are listed in Table 1. All bacterial cultures were grown in Luria Bertani (LB) medium (Miller 1972) which was solidified with 1.5% bacteriological agar (Gibco BRL) when required. Antibiotics were included in the growth medium at the following concentrations: ampicillin (Ap) 100 μg/mL; kanamycin (Km) 50 μg/mL; rifampicin (Rf) 50 μg/mL; tetracycline (Tc) 15 μg/mL; gentamicin (Gm) 15 μg/mL. Methyl methanesulphonate (MMS) was used at 200 μg/mL and mitomycin C (Sigma) was used at 0.5 μg/mL. *Escherichia coli* strains were grown at 37°C and *P. aureofaciens* at 30°C.

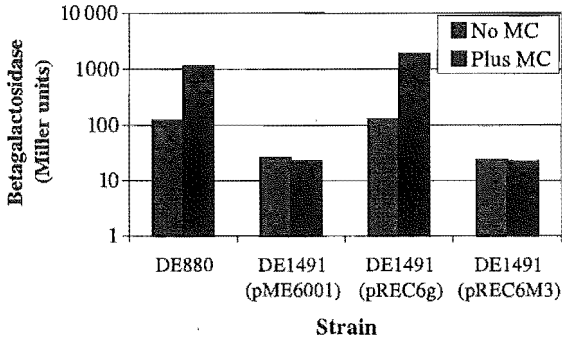
Isolation and subcloning of MMS resistance-encoding cosmids

The ability to provide *recA* defective *E. coli* strains with resistance to the DNA-damaging agent MMS has previously been associated with complementation by *recA*$^+$ clones (Better and Helinski 1983). The genomic library of PA147-2 (stored in *E. coli* DH5α) was plated on LB plates supplemented with tetracycline and MMS. Six colonies grew after overnight incubation, and these were all shown to contain identical cosmids (data not shown). One of the cosmids (pREC1) was used to transform a *recA* mutant of *E. coli* MC4100 to MMS resistance, thus confirming the ability to confer MMS resistance. A 6kb *Eco*RI/*Hin*dIII fragment that encodes MMS resistance was cloned into the vector pME6001, creating pREC6g(*recA*$^+$). A *recA* mutant of pREC6g(*recA*$^+$), called pREC6M3(*recA*), was created by insertional mutagenesis with miniTn*10*, as previously described (Kleckner et al. 1991).

Induction of the SOS response in *Escherichia coli*

RecA plays a critical role in the SOS response in *E. coli*, acting as a catalyst in cleavage of the LexA protein and thus derepressing the SOS regulon. Expression of *sulA* is induced in *E. coli* by the SOS response, and SulA arrests cell division through interaction with FtsZ (Bi and Lutkenhaus 1993). We tested the ability of the PA147-2 *recA* to induce SOS induction by measuring expression of the *sulA* gene in *E. coli* strains DE1491(*recA*) and DE880(*recA*$^+$). DE1491

Fig. 1. Induction of the SOS response in *E. coli* DE1491(*recA*) and DE880(*recA*$^+$). The β-galactosidase level from DE1491 with each of the plasmids shown was measured, with and without mitomycin C (MC) added to the cultures. The *recA*$^+$ strain DE880 was included as a control. Both *E. coli* DE1491 and DE880 have a *sulA::lacZ* fusion, so β-galactosidase levels reflect the level of SulA, which is induced in the SOS response.



and DE880 have a Mud*lac* insertion into *sulA*, creating an in-frame *sulA::lacZ* fusion. The expression of *sulA* can therefore be inferred by measuring the β-galactosidase level according to the method of Miller (1972). When pREC6g(*recA*$^+$) was present in DE1491, an increase in *sulA* expression was observed, relative to DE1491 with either pME6001 or pREC6M3(*recA*). The expression of *sulA* was further increased when the SOS response was induced by exposure to the DNA-damaging agent mitomycin C for 60 min prior to the β-galactosidase assay. In contrast to these observations, the presence of either pME6001 or pREC6M3(*recA*) in DE1491 had no impact on *sulA* expression with or without addition of mitomycin C (Fig. 1). When *E. coli* DE880, an otherwise isogenic *recA*$^+$ strain of DE1491, was assayed under the above conditions, the level of β-galactosidase produced after mitomycin C exposure was comparable to that from DE1491(pREC6g(*recA*$^+$)) (Fig. 1).

Resistance to DNA damage induced by UV and MMS

Escherichia coli strains DH5α and MC4100 are sensitive to exposure to UV and MMS, due to DNA damage. Resistance to UV and MMS was assessed qualitatively by the ability of bacterial strains to survive treatment with these DNA damaging agents. When pREC6g(*recA*$^+$) was present in trans, both strains showed an increased resistance to UV and MMS. However, this increase in resistance was not detected when pREC6g(*recA*$^+$) was replaced with either pME6001 or pREC6M3(*recA*), indicating a role for the PA147-2 *recA* gene in the provision of UV and MMS resistance, presumably by the restoration of RecA-dependent DNA-damage repair systems. *Pseudomonas aureofaciens* PA147-2 *recA* mutants (described below) showed increased sensitivity to both UV and MMS, and resistance was restored by in trans complementation with pREC6g(*recA*$^+$), but not with pME6000(vector) or pREC6M3(*recA*::Tn*10*) (data not shown).

Table 2. Promotion of homologous recombination.

Strain	Inheritance of *his*[a]	Inheritance of *ilv*[a]
MT2 (pREC6g)	3.7×10^{-6}	9.5×10^{-5}
MT2 (pREC6M3)	$<2.6 \times 10^{-7}$	$<2.6 \times 10^{-7}$
MT2 (pME6001)	$<1.8 \times 10^{-7}$	$<1.8 \times 10^{-7}$

Note: Homologous recombination is inferred from the frequency of inheritance.

[a]Inheritance is expressed as the number of cells inheriting the particular allele out of the total population.

Promotion of homologous recombination by pREC6g(*recA*$^+$)

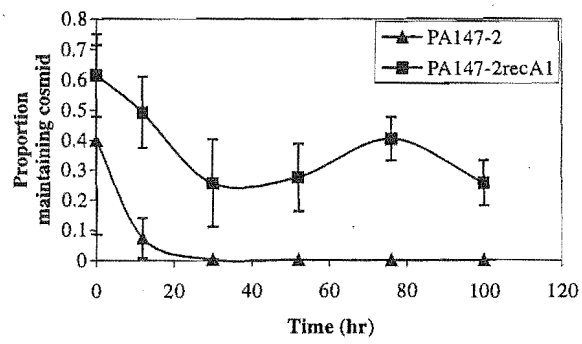
The ability of the MMS resistance-encoding clone pREC6g(*recA*$^+$) to promote homologous recombination was tested by P1 transduction of functional amino acid biosynthetic *his* and *ilv* alleles to a *his ilv E. coli* mutant (*E. coli* MT2). $P1_{vir}$ stock was grown on *E. coli* W3110, and used to transduce the auxotrophic *E. coli* strains by the method of Miller (1972). Inheritance of the nutritional marker was used to indicate recombination between the transduced DNA and the genome of the recipient. Strains (*E. coli* MT2) containing pREC6g(*recA*$^+$) showed inheritance of transduced markers whereas transductants of the control strains bearing either pME6001 or pREC6M3(*recA*) were not detected (Table 2). No spontaneous revertants of MT2 to either His$^+$ or Ilv$^+$ were detected.

Our primary motivation for this study was to investigate the possibility that disruption of *recA* in PA147-2 might alleviate the problem of recombination during complementation experiments. Since genetic systems are not well developed in *P. aureofaciens*, we used an *E. coli* system to test homologous recombination frequency. Our data show that the wild-type *recA* clone of PA147-2 (pREC6g(*recA*$^+$)) can promote homologous recombination in *E. coli*, and an insertional mutant (pREC6M3(*recA*)) was unable to promote this RecA-dependent function.

Introduction of *recA* mutations to the chromosome of PA147-2

The cosmid pREC1, which carries *recA*$^+$ derived from PA147-2, was subjected to insertional mutagenesis with miniTn*10* (Kleckner, et al. 1991). Insertions in *recA* were identified by assaying for a loss of MMS resistance when the mutated cosmid was in an *E. coli* DH5α background. Two independent mutants were recovered and designated pPRAM1 and pPRAM4. These plasmids were transferred to PA147-2 by conjugation, and the mutated *recA* alleles were introduced to the genome of PA147-2 by homologous recombination. This resulted in the strains PA147-2recA1 and PA147-2recA4, both of which exhibit a loss of resistance to UV irradiation and MMS exposure. Recombination was indicated by the loss of the vector marker (Tc resistance) and the inheritance of MMS sensitivity and Km resistance (from the *recA*::miniTn*10*), and was subsequently confirmed by Southern analysis (data not shown). The two mutant cosmids pPRAM1 and pPRAM4 were confirmed as having different insertion points by DNA sequencing using a primer designed to anneal to the IS*10* of the miniTn*10* element (the IS*10* primer sequence is 5′-GGGATCATATGACAAGATGT-3′). In

Fig. 2. Stability of cosmid pPS2122 in PA147-2, and PA147-2recA1. The proportion with the cosmid was determined by dividing the tetracycline (Tc)-resistant cfu·mL^{-1} by the total cfu·mL^{-1} at each given time point. Tc resistance is conferred by the cosmid, and hence Tc-sensitive colonies arise from cells that have lost the cosmid. Data presented are the average of three independent experiments and are shown with the standard error.



addition to demonstrating the different mutant insertion points, the DNA sequence data showed greater than 90% similarity to *recA* sequences from other *Pseudomonas* spp. (data not shown).

Stability of cosmids in PA147-2 and *recA* deficient derivatives

Since we were interested in creating a system in which cosmids could be used to complement antifungal mutants in trans, we examined the stability of a cosmid in PA147-2 and the *recA* mutant derivatives PA147-2recA1 and PA147-2recA4. The cosmid pPS2122 is a pLAFR3-based cosmid thought to contain at least one gene of importance in antifungal activity and has been shown to restore antifungal activity to *P. aureofaciens* PA138, a Tn*5* mutant of PA147-2, by allele exchange (Silby and Mahanty 1999*a*). However, attempts at complementation in trans revealed that pPS2122 was unstable in PA138 if selection for the cosmid was not maintained. Cosmid pPS2122 was introduced to PA147-2, PA147-2recA1, and PA147-2recA4 by conjugation, and maintenance of pPS2122 was tested by growing transconjugants in 10 mL LB cultures without selection for the cosmid. Samples of the culture were taken periodically, serially diluted, and plated on LB agar supplemented with Rf and LB agar plus Rf and Tc. Total colony forming unit counts on Rf selection reflect the total number of culturable cells, whereas only those maintaining the cosmid could grow in the presence of Tc. Our results indicate that pPS2122 was considerably more stable in PA147-2recA1 than in the wildtype (Fig. 2), whereas there was little difference in stability between PA147-2recA4 and PA147-2.

We have shown that the stability of a pLAFR3-based cosmid is increased in PA147-2recA1, but is not significantly improved in PA147-2recA4. This is an interesting finding given that very few studies on *recA* have reported similar effects on plasmid stability. An analysis of the archael *recA* analog, *radA*, indicates that *radA* is involved in the maintenance or replication of some halobacterial plasmids in *Haloferax volcanii* (Woods and Dyall-Smith 1997), while a recent report suggests that plasmid stability can be reduced in *recA* mutants of *E. coli* (Debbia 1992). These studies demonstrate reduced maintenance of plasmids in *recA/radA* mutants. In contrast, the cosmid pPS2122 shows increased stability in PA147-2recA1, but not in PA147-2recA4. The transposon insertion points in these mutants have been mapped to near the middle of *recA* (PA147-2recA1) and toward the 3′ end (PA147-2recA1). These data suggest that RecA is involved in the instability of pPS2122 in PA147-2, and that the insertion in PA147-2recA4 reduces this effect. It is possible that the domain of RecA responsible for the plasmid instability is encoded upstream of the insertion in PA147-2recA4, and thus it is still expressed in the mutant. In this case it is tempting to suggest that the role of RecA in plasmid instability is genetically separable from its role in DNA repair since both *recA* strains show increased sensitivity to DNA-damaging agents, but only one has a change in plasmid stability. In PA147-2recA1, the insertion point is near the middle of the gene, which appears to be sufficient to abolish the instability phenotype associated with pPS2122 in PA147-2. One possible role for RecA in cosmid stability is cosmid loss during homologous recombination between the cosmid clone and the host genome. However, similar experiments using the cosmid vector pLAFR3 with no PA147-2 genomic insert give similar results to those described here, making the recombination hypothesis unlikely (Silby and Mahanty 1999*b*). It is possible that recombination between pLAFR3 molecules or formation of multimers could lead to the instability, but this has yet to be tested. Further mutagenesis may uncover the important region in RecA that leads to the observed phenotype, and a complete DNA sequence of *recA* will aid in the analysis of the mutants. Regardless of the molecular basis for the observation, the increased stability of pPS2122 in PA147-2recA1 strongly suggests that the creation of *recA* strains will be important in in trans complementation analysis of antifungal minus mutants of PA147-2. Further studies will test the stability of additional cosmids, and the necessity to develop a simple system for the creation of unmarked *recA* mutants in PA147-2 and its Tn*5* mutant derivatives.

In summary, we have shown the isolation and functional analysis of *recA* from *Pseudomonas aureofaciens* PA147-2, and the creation of *recA* mutants of PA147-2. The PA147-2 *recA* can restore RecA-dependent phenotypes in *E. coli recA* strains, and *recA* mutants of PA147-2 show increased sensitivity to MMS and UV compared to the wild type. With regard to our original aim of improving complementation in PA147-2 and mutants lacking antifungal activity, we have shown that (*i*) cosmids are more stable in *recA* mutants of PA147-2 than the wildtype, and (*ii*) a mutant *recA* allele from PA147-2 cannot promote homologous recombination in *E. coli*. The combination of these two effects will help in the analysis of antifungal genes by complementation in *recA*-defective strains of PA147-2 and antifungal minus mutant derivatives.

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References

Ball, T.K., Wasmuth, C.R., Braunagel, S.C., and Benedik, M.J. 1990. Expression of *Serratia marcescens* extracellular proteins requires *recA*. J. Bacteriol. **172**: 342–349.

Better, M., and Helinski, D.R. 1983. Isolation and characterisation of the *recA* gene of *Rhizobium meliloti*. J. Bacteriol. **155**: 311–316.

Bi, E., and Lutkenhaus, J. 1993. Cell division inhibitors SulA and minCD prevent formation of the FtsZ ring. J. Bacteriol. **175**: 1118–1125.

Carruthers, F.L., Conner, A.J., and Mahanty, H.K. 1994. Identification of a genetic locus in *Pseudomonas aureofaciens* involved in fungal inhibition. Appl. Environ. Microbiol. **60**: 71–77.

Carruthers, F.L., Shum-Thomas, T., Conner, A.J., and Mahanty, H.K. 1995. The significance of antibiotic production by *Pseudomonas aureofaciens* PA147-2 for biological control of *Phytophthora megasperma* root rot of asparagus. Plant Soil, **170**: 339–344.

Clark, A.J., and Margulies, A.D. 1965. Isolation and characterisation of recombination-deficient mutants of *E. coli* K12. Proc. Natl. Acad. Sci. U.S.A. **53**: 451–459.

De Mot, R., Laeremans, T., Schoofs, G., and Vanderlayden, J. 1993. Characterisation of the *recA* gene from *Pseudomonas fluorescens* OE 28.3 and construction of a *recA* mutant. J. Gen. Microbiol. **139**: 49–57.

Debbia, E.A. 1992. Filamentation promotes F'*lac* loss in *Escherichia coli* K12. J. Gen. Microbiol. **138**: 2083–2091.

Ennis, D.G., Ossanna, N., and Mount, D.W. 1989. Genetic separation of *Escherichia coli recA* functions for SOS mutagenesis and repressor cleavage. J. Bacteriol. **171**: 2533–2541.

Gilson, L., Mahanty, H.K., and Kolter, R. 1987. Four plasmid genes are required for colicin V synthesis, export and immunity. J. Bacteriol. **169**: 2466–2470.

Hanahan, D. 1983. Studies on transformation of *Escherichia coli* with plasmids. J. Mol. Biol. **166**: 557–580.

Kleckner, N., Bender, J., and Gottesman, S. 1991. Uses of transposons with an emphasis on Tn*10*. Methods Enzymol. **204**: 139–180.

Kohara, Y., Akiyama, K., and Isono, K. 1987. The physical map of the whole *E. coli* chromosome: Application of a new strategy for rapid analysis and sorting of a large genomic library. Cell, **50**: 495–508.

Kowalczykowski, S.C., Dixon, D.A., Eggleston, A.K., Lauder, S.D., and Rehrauer, W.M. 1994. Biochemistry of homologous recombination in *Escherichia coli*. Microbiol. Rev. **58**: 401–465.

Luo, J., Burns, G., and Sokatch, J.R. 1993. Construction of chromosomal *recA* mutants of *Pseudomonas putida* PpG2. Gene, **136**: 263–266.

Miller, J.H. 1972. Experiments in molecular genetics. Cold Spring Harbour Laboratory Press, Cold Spring Harbour, New York.

Miller, R.V., and Kokjohn, T.A. 1990. General microbiology of *recA*: Environmental and evolutionary significance. Annu. Rev. Microbiol. **44**: 365–394.

Salles, B., Weisemann, J.M., and Weinstock, G.M. 1987. Temporal control of colicin E1 induction. J. Bacteriol. **169**: 5028–5034.

Sano, Y., and Kageyama, M. 1987. The sequence and function of the *recA* gene and its protein in *Pseudomonas aeruginosa* PAO. Mol. Gen. Genet. **208**: 412–419.

Silby, M.W., and Mahanty, H.K. 1999*a*. (University of Canterbury). Unpublished data.

Silby, M.W., and Mahanty, H.K. 1999*b*. (University of Canterbury). Unpublished data.

Silhavy, T., Berman, M., and Enquist, L. 1984. Experiments with gene fusions. Cold Spring Harbour Laboratory Press, Cold Spring Harbour, N.Y.

Simon, R., Priefer, U., and Puhler, A. 1983. A broad host range mobilisation system for in vivo engineering: Transposon mutagenesis in gram-negative bacteria. Biotechnology, **1**: 748–751.

Toman, Z., Dambly-Chaudiere, C., Tenenbaum, L., and Radman, M. 1985. A system for detection of genetic and epigenetic alterations in *Escherichia coli* induced by DNA-damaging agents. J. Mol. Biol. **186**: 97–105.

Willis, D.K., Hrabak, E.M., Lindow, S., and Panopoulos, N.J. 1988. Construction and characterisation of *Pseudomonas syringae recA* mutant strains. Mol. Plant Microb. Interact. **1**: 80–86.

Woods, W.G., and Dyall-Smith, M.L. 1997. Construction and analysis of a recombination-deficient (*radA*) mutant of *Haloferax volcanii*. Mol. Microbiol. **23**: 791–797.

Statement on my contributions to published work

Godfrey et al (2000) was jointly authored by Scott Godfrey and myself. In it we report the results of a field trial which we undertook together. I contributed equally to the design and execution of the trial. Mr Godfrey did 75% of the work on bacterial isolation, while I carried out the molecular analysis. Overall, I contributed 50% to this paper.

Silby and Mahanty (2000) was written by me, with advice from my supervisor Associate Professor H. Khris Mahanty. I did 100% of the work reported.

Mark W. Silby, 13 November 2001